

Variant Estrogen Receptor mRNA Species Detected in Human Breast Cancer Biopsy Samples

Leigh C. Murphy* and Helmut Dotzlaw

Department of Biochemistry
University of Manitoba
Winnipeg, Manitoba, Canada, R3E 0W3

Thirty to 40% of estrogen receptor (ER) positive human breast tumors are resistant to endocrine therapy. To investigate the possibility that abnormal ER proteins may be associated with this endocrine resistant phenotype we have looked for the presence of abnormally sized ER mRNA in human breast tumor biopsies.

Poly(A⁺) enriched RNA was isolated from 46 human breast tumor biopsies and analyzed by Northern blotting and hybridization with the human estrogen receptor OR-8 cDNA. Seventy percent of the tumors contained detectable 6.5 kilobase (kb) ER mRNA. Some tumors also contained smaller sized ER mRNA of approximately 3.8 and 2.4 kb. These variant sized transcripts were only detected in biopsies where the normal 6.5 kb mRNA was present. In some cases the abundance of the variant mRNAs was equal to or greater than the normal ER mRNA. The variant ER mRNAs were not due to nonspecific RNA degradation nor was there any evidence of gross alteration of the ER gene in tumors where variant mRNAs were detected.

ER cDNA fragments representing only the E/F domain of the receptor showed little or no hybridization with the variant sized ER mRNAs, while a ER cDNA fragment covering the A/B, C and D domains hybridized to both the variant and normal ER mRNAs. This suggested that the smaller variant ER mRNAs may be missing some or all of the E/F domain which is thought to contain the steroid hormone binding domain of the receptor.

It is hypothesized that if these intermediates were translated into viable proteins they may interfere with the normal ER function. (*Molecular Endocrinology* 3: 687-693, 1989)

INTRODUCTION

At presentation only 30-40% of human breast cancers are hormonally dependent, that is they will respond to

some form of endocrine therapy, e.g. ovariectomy, hypophysectomy, antiestrogen, or high dose estrogen therapy. However, the majority of human breast cancers originate as hormonally dependent tumors since ovariectomized, nonestrogenized women (Turner's syndrome) and males rarely develop breast cancer (1). While the mechanisms responsible for hormone dependence in human breast cancer are complex and poorly understood, the likelihood of response to endocrine therapy, e.g. the antiestrogen tamoxifen, correlates strongly with the presence of estrogen receptors (ER) (2). Although our previous studies (3) and those from other laboratories have identified an additional antiestrogen binding site, direct competition of tamoxifen with estrogen for binding to the estrogen receptor (4) is thought to be the basis of its antiestrogenic and antitumor activity. While tamoxifen is an extremely useful agent in the treatment of breast cancer it is rarely if ever curative since there is, almost invariably, the development of resistance to tamoxifen in the breast tumors of those patients who had previously responded. Furthermore, there is a large subset (~30-40%) of ER-positive patients who fail to respond to this agent (5). In some cases tamoxifen resistance may be due to the selection of a hormonally independent clone, i.e. ER-negative, from an initially heterogeneous tumor, but in other cases the tumor remains ER positive and interestingly, will often subsequently respond to another form of hormonal manipulative therapy such as progestin, stilbestrol, chemical, or surgical adrenalectomy (6).

The cDNA cloning of many members of the steroid hormone receptor family has led to the observation that this gene family displays sequence similarity to v-erb A, a truncated form of c-erb A which encodes the thyroid hormone receptor. The protein product of v-erb A does not bind ligand and it has been suggested that its ability to potentiate the transforming activity of v-erb B is due to it being a T₄-independent transcription factor (7, 8). Moreover, deletion mutants of the glucocorticoid receptor which no longer bind hormone have been shown to be constitutive transcription activators (9, 10). Similar studies with the estrogen receptor have shown that deletion of most or all of the hormone binding domain leads to constitutive transcriptional activity, albeit markedly reduced, when compared to the intact receptor.

These mutants, however, were still efficient in binding to estrogen responsive elements (11). These studies suggest that steroid hormones may not be essential to achieve receptor-mediated gene expression and therefore the presence of other forms of receptors may be mechanistically associated with the development of hormone independence (12) and androgen resistance. The data presented here support the possibility that variant sized ER species for the ER are present in some human breast cancer biopsy samples.

RESULTS

Northern Analysis of RNA Isolated from Human Breast Tumor Biopsy Samples

A total of 46 human breast cancer biopsy specimens of sufficient size (0.8–2 g) such that enough RNA could be isolated for subsequent poly(A⁺) enrichment was subjected to Northern blot analysis. Hybridization of the resulting blot with a human ER cDNA [OR-8 (12)] demonstrated the presence of one major mRNA of approximately 6.5 kilobases (kb) in 70% of the breast tumors analyzed. The presence of this transcript in the main correlated with the presence of ER as determined by classical binding assays. However, two tumors were found to be negative by binding assay (<3 fmol/mg protein) but contained detectable ER mRNA and one tumor was borderline ER positive (3.7 fmol/mg protein) by binding assay but did not contain ER mRNA. Surprisingly, in some tumors the OR-8 ER cDNA probe was found to also hybridize with mRNA species which were of smaller size (Fig. 1). The two most commonly occurring variant sized transcripts were of approximately 3.8 and 2.4 kb. Occasionally a 4.5-kb transcript was also observed. While a minor transcript of about 4 kb can also be seen together with the 6.5 kb transcript in ER positive breast cancer cell lines (13, 13a), we and others have not observed the other variant sized transcripts in breast cancer cell lines (13–14) or human endometrium (14). Examples of tumor RNA containing normal and variant sized estrogen receptor mRNA are presented in Figs. 1A and 2A. The presence of variant sized ER mRNAs was not associated with any obvious nonspecific degradation of RNA as shown by ethidium bromide staining (Fig. 1C and 2B) and the ability to detect by specific hybridization the 5 kb epidermal growth factor (EGF) precursor RNA on the same blots (Fig. 1B). Hybridization of the OR-8 cDNA probe with the blots using more stringent (50°C, 50% formamide) conditions yielded the same results. This suggests that the smaller sized ER mRNA species were unlikely to be due to cross-hybridization with another similar but unique member of the ERB-A family.

When the 46 tumors were divided into low ER binding (<20 fmol/mg protein) and high ER binding (≥20 fmol/mg protein) 33% and 60%, respectively, contained variant sized ER mRNAs. It should be emphasized, however, that variant sized ER mRNAs were detected only

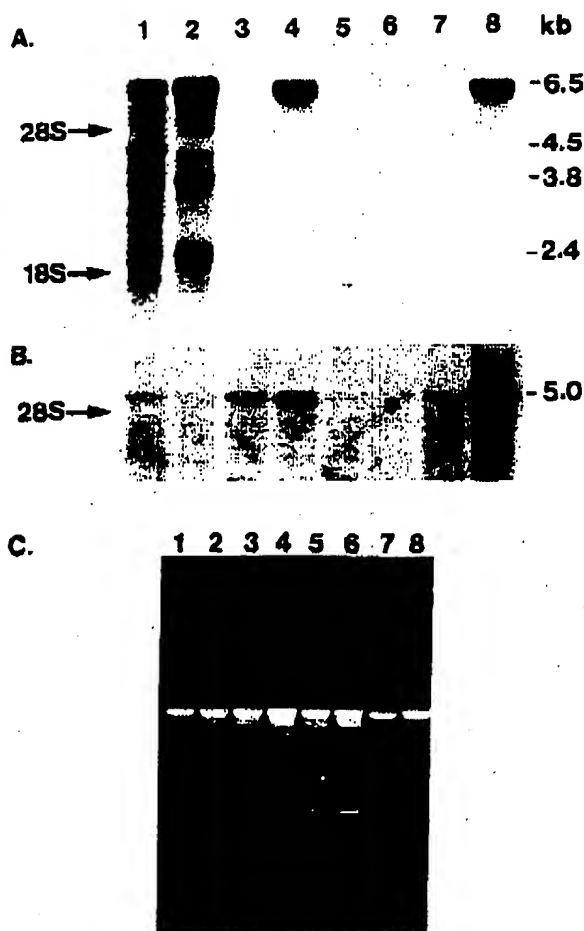


Fig. 1. Northern Analysis of Poly(A⁺) Enriched RNA Isolated from Some Human Breast Tumor Biopsies

A. Twenty micrograms of poly(A⁺) enriched RNA isolated individually from eight human breast tumor biopsy samples was analyzed by Northern blotting. The blot was hybridized with the human ER cDNA OR-8 and the results visualized by exposure to x-ray film overnight with an intensifying screen. Arrows show the positions of the residual 28S and 18S ribosomal RNA as indicated. The calculated size of each hybridizing band is shown on the right-hand side of the diagram. B. The same blot as above hybridized with the human EGF cDNA. C. Ethidium bromide staining of the above RNA gel before transfer to the nitrocellulose filter.

In biopsies where the normal 6.5 kb mRNA was present. On no occasion have we observed variant sized transcripts in the absence of the normal transcript. However, in some tumors as shown in Fig. 2A, lanes 3 and 5, the variant sized transcript or transcripts are as abundant or more abundant than the normal 6.5 kb transcript.

Structural Analysis of Variant Sized ER mRNAs in some Human Breast Tumor Biopsy Samples

To further understand the structure of the smaller ER mRNAs in relation to the 6.5 kb mRNA, the OR-8 cDNA was subcloned into two fragments as shown in Fig. 3, the J fragment representing the regions encoding the

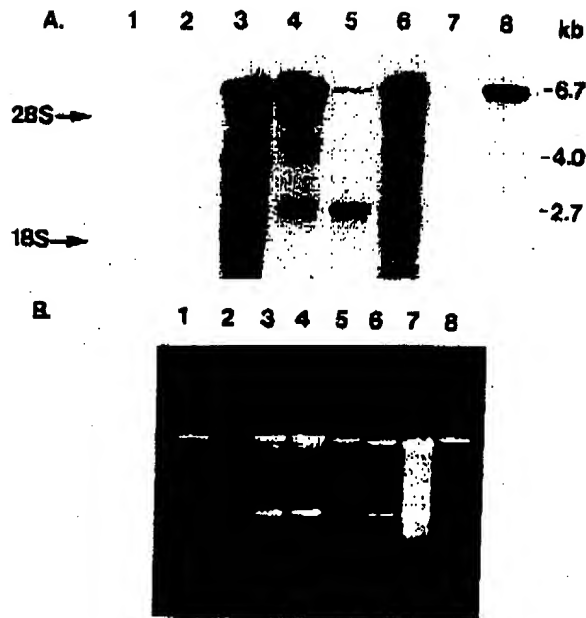


Fig. 2. Northern Analysis of Poly(A⁺) Enriched RNA Isolated from Another Series of Human Breast Tumor Biopsies

A, Thirteen micrograms of poly(A⁺) enriched RNA isolated individually from another group of seven human breast tumor biopsies (lanes 1-7) and T-47D cells (lane 8) was analyzed by Northern blotting. The blot was hybridized with the human ER cDNA OR-8 and the results visualized by exposure to x-ray film for 24 h with an intensifying screen. Arrows show the positions of the residual 28S and 18S ribosomal RNA as indicated. The calculated size of each hybridizing band is shown on the right-hand side of the diagram. B, Ethidium bromide staining of the above RNA gel before transfer to the nitrocellulose filter.

A/B, C, and D domains of the receptor and O fragment representing primarily the regions encoding the E and F domains of the receptor. Although the J fragment hybridized to all ER transcripts the O fragment showed reduced or no hybridization to the smaller variant sized estrogen receptor transcripts (Figs. 4 and 5). This suggested that the smaller transcripts may be missing some or all of that area encoding the E/F domain which is thought to contain the steroid hormone binding domain of the ER.

Structural Analysis of the ER Gene in Some Human Breast Tumor Biopsy Samples

The structure of the ER gene in tumors showing normal, normal plus variant sized, and no ER mRNA was inves-

tigated next. DNA was isolated from two to three samples of each of the tumor categories stated above and analyzed by Southern blotting. The results of *PvuII* and *PstI* restriction digests are shown in Fig. 6, A and B, respectively. No deletions or gross rearrangements could be observed in the ER gene in this series of tumors. The *PvuII* restriction enzyme digest confirmed the presence of six invariant restriction fragments [19.5, 7.24, 5.75, 3.89, 3.24, and 1.07 kilobase pairs (kbp)] plus the presence of a restriction fragment length polymorphism (RFLP) in two other bands of 1.74 and 0.78 kbp (16). This RFLP was not correlated with the presence or absence of variant sized ER mRNA although in this small group of tumors it was associated with little or no ER binding activity. The ER content of each tumor (1-9) as measured by classical binding assay was 19.4, 6.6, 3.3, 9.4, 39, 55, 21, 1.1, 0.8 fmol/mg protein, respectively. The tumors which were homozygous for the 0.78 kbp RFLP (tumor 3, 8, and 9) were either ER negative or at the borderline for ER positivity (ER positivity defined as >3 fmol/mg protein). This is consistent with the observations made by Hill *et al.* (15).

DISCUSSION

The hypothesis that altered forms of steroid hormone receptor proteins may be associated with the hormone independent and/or resistant phenotype is an attractive one. Previously altered forms of glucocorticoid receptors have been found associated with glucocorticoid resistance in some mouse lymphoma cell lines (16, 17) and point mutations in the 1,25-dihydroxyvitamin D receptor have been found to be associated with clinical type II vitamin D resistance rickets (18). Interestingly, point mutations, detected by RNase protection assay, in ER mRNAs obtained from some human breast tumor biopsies have recently been described (19) and these mutations are in some cases associated with discrepancies in the presence of ER mRNA and ER as measured by classical binding assay. It has been recently reported in abstract form (20) that although there is very little, if any, estrogen binding in the estrogen independent and androgen resistant T-47D⁺ human breast cancer cell line (21) there is still a substantial amount (approximately 50% that seen in MCF 7 cells) of ER mRNA. It was therefore speculated that the estrogen resistance in these cells might be due to the production of a

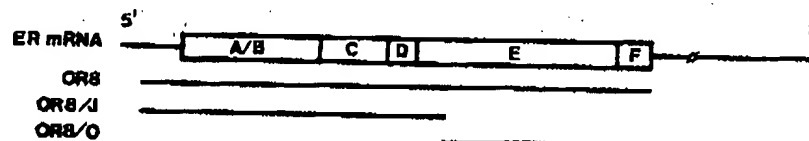


Fig. 3. The Human ER mRNA and the Corresponding cDNA Probes Used in this Study

Schematic representation of the human ER mRNA. The open box represents the coding region which has been divided into domains A-F (11, 12). The ER cDNA fragments used in this study are shown. A convenient *HindIII* restriction site in the 5'-end of the E domain was used to divide the OR-8 cDNA into the two fragments J and O.

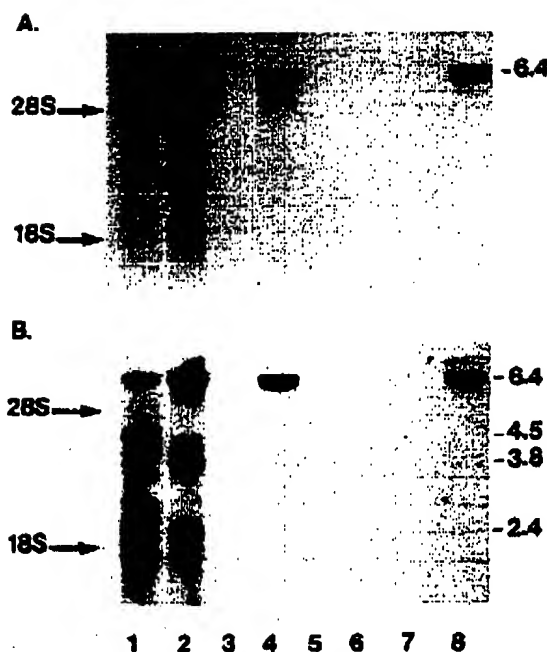


Fig. 4. Differential Hybridization of OR-8/J and OR-8/O ER cDNA Fragments to Normal and Variant ER mRNA

A, The same blot as in Fig. 1 hybridized with OR-8/O ER cDNA. B, The same blot hybridized with OR-8/J ER cDNA. All autoradiograms shown were overnight exposures in the presence of an intensifying screen. Arrows show the positions of the residual 28S and 18S ribosomal RNA as indicated. The calculated size of each hybridizing band is shown on the right-hand side of the diagram.

defective receptor protein (20). Moreover, a truncated nonligand binding form of the thyroid receptor, the v-erb A protein, has been associated with transformation in avian erythroblastosis (7, 8) and a truncated nonligand binding form of the EGF receptor has also been identified in A431 human vulval carcinoma cells (22), although the significance of its production to the transformed phenotype, if any, is not known. Much of this evidence although still somewhat preliminary does point to the presence of a variety of physically altered forms of receptors which may be mechanistically involved in hormone independence and resistance.

Although the hormone-dependent phenotype in human breast cancer is highly associated with the presence of ER in the tumor, at presentation 30–40% of ER positive tumors do not respond to hormonal manipulative therapy and are therefore estrogen independent and antiestrogen resistant. Moreover, the majority of tumors which previously responded to antiestrogen therapy will eventually develop resistance to this treatment without necessarily altering the ER profile (5). The presence of altered forms of receptor proteins in these tumors might be a possible mechanism associated with this resistant phenotype. Cloning of the ER has shown that in normal target tissues only one approximately 6.5 kb mRNA encodes the ER protein (23). We are

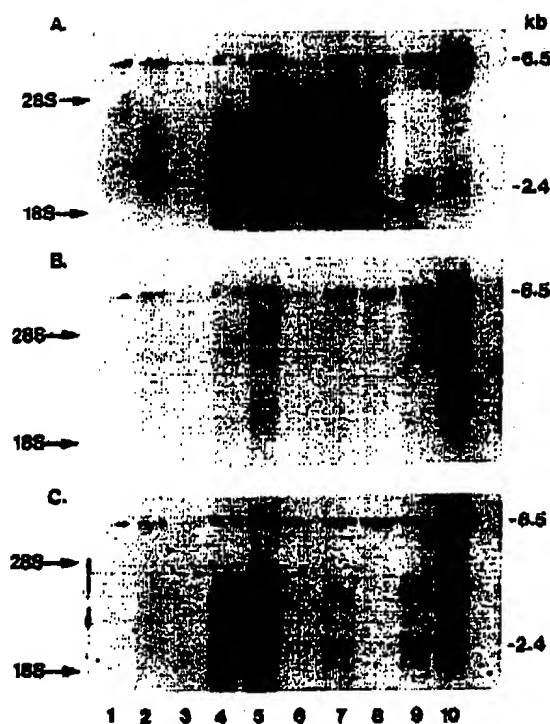


Fig. 5. Differential Hybridization of OR-8/J and OR-8/O ER cDNA Fragments to Normal and Variant ER mRNA

A, Ten micrograms of poly(A)⁺ RNA was isolated from T-47D (lane 1), MCF 7 (lane 2), a reduction mammioplasty sample (lane 3), and another group of seven (lanes 4–10) human breast biopsy samples and analyzed by Northern blotting. The blot was hybridized with the human ER cDNA OR-8 and the results visualized by exposure to x-ray film for 2 days with an intensifying screen. Arrows show the positions of the residual 28S and 18S ribosomal RNA as indicated. The calculated size of each hybridizing band is shown on the right-hand side of the diagram. B, The same blot as above hybridized with the OR-8/O ER cDNA and the results visualized by exposure to x-ray film for 3 days with an intensifying screen. C, The same blot as above hybridized with the OR-8/J ER cDNA and the results visualized by exposure to x-ray film for 3 days with an intensifying screen.

investigating the possibility that putative altered ER proteins may be encoded by altered ER mRNAs. Initially, we have looked for altered sizes of ER mRNAs by Northern analysis of poly(A)⁺ enriched RNA isolated from a range of human breast cancer biopsy samples. In some of these tumors we have detected smaller sized ER mRNAs in the presence of the normal 6.5 kb mRNA. The smaller sized transcripts may be due to use of alternative polyadenylation signals shown to be present in the ER gene (12) or different lengths of poly(A) tails, our data do not exclude these possibilities. Our data do suggest, however, that these smaller sized transcripts are missing a substantial portion of the E/F coding region. At this stage we do not have the appropriate probes to investigate the 3'-untranslated region of these transcripts; therefore, we cannot rule out the possibility that some of this area may be missing as

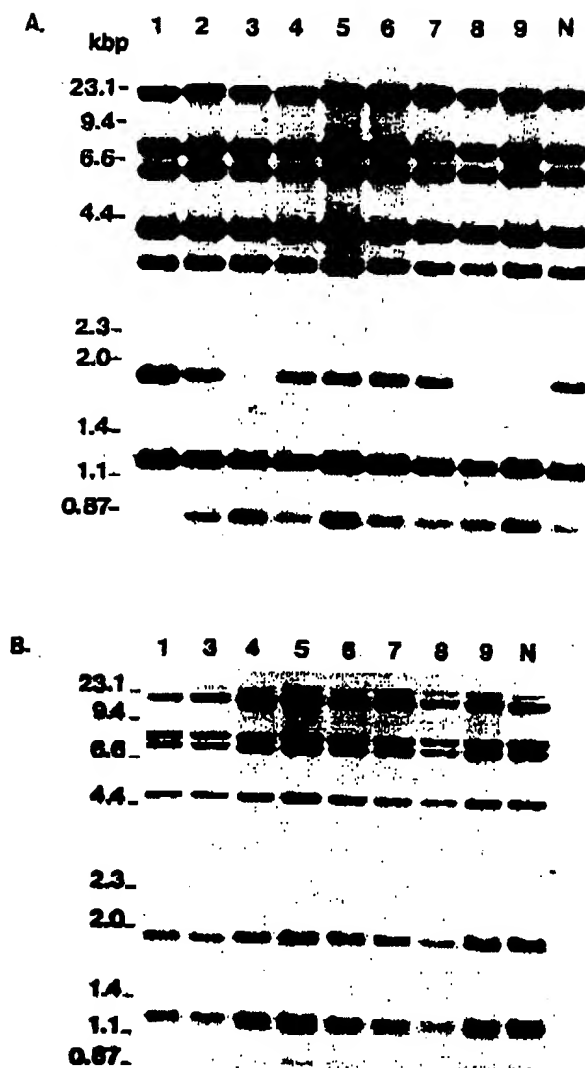


Fig. 6. Southern Analysis of DNA Isolated from Some Human Breast Tumor Biopsies

A. DNA was isolated from tumors containing normal ER mRNA (lanes 1-3), normal and variant ER mRNA (lanes 4-7), no ER mRNA (lanes 8-9), and normal human lymphocytes (lane N) as described in *Materials and Methods*. Twelve and a half micrograms of each were digested with *PvuII* and analyzed by Southern blotting. Blots were hybridized with OR-8 ER cDNA and the results visualized by exposure to x-ray film for 3 days plus an intensifying screen. The calculated restriction fragment sizes are shown to the left of the diagram. B. Another aliquot of DNA from the above tumors (except for tumor 2) was digested with *PstI* and analyzed as described above, except that the results were obtained after overnight exposure of the blot to x-ray film plus an intensifying screen. Lane 1 is DNA isolated from tumor shown in Fig 5, lane 8. Lane 3 is DNA isolated from tumor shown in Fig 5, lane 6. Lane 5 is DNA isolated from tumor shown in Fig 2, lane 4. Lane 6 is DNA isolated from tumor shown in Fig 2, lane 3. Lane 9 is DNA isolated from tumor shown in Fig. 2 lane 2. In many cases there was no tumor remaining after RNA isolation to allow for DNA isolation.

well. It could be speculated that if these smaller sized mRNAs were able to be translated then the protein product would be an ER protein which was missing a substantial portion of the hormone binding domain. It is known that *in vitro* mutated forms of the ER protein missing the hormone binding domain can still interact with estrogen responsive elements and in some cases maintain a degree of constitutive enhancer activity (11). In the cases where we have observed that the smaller sized transcripts are more abundant than the normal transcript, it is possible that the variant protein products might seriously compete with the normal protein for regulatory sites in the genome of these tumor cells.

The origin of these smaller sized ER transcripts is unknown. It does not appear to be due to nonspecific degradation of the RNA. Nor is there any evidence of gross alteration of the ER gene in tumors where variant mRNAs were detected. More subtle mutations in the gene may lead to alternatively spliced transcripts, or some alteration of the processing steps involved in the production of the mature mRNA. Alternatively, the normal processes involved in the turnover and degradation of ER mRNA may be altered in some tumors resulting in an accumulation of degradation intermediates. These intermediates if translated into a viable protein may interfere with the normal ER protein function. At present we have no evidence which supports an association of these smaller ER transcripts with estrogen independent or tamoxifen resistant tumors.

The relationship of the variant sized ER mRNAs to neoplastic tissue is unknown. Interestingly, in the one reduction mammoplasty sample we have been able to analyze (see Fig. 5, lane 3) a normal 6.5 kb ER mRNA was detected at low abundance, while no smaller ER transcripts were detectable. The significance of this observation is unclear since the abundance of the 6.5 kb ER mRNA in this sample is low and the lack of detection of smaller ER transcripts may be due merely to their falling below the limit of detection of the assay. The presence or absence of altered sized ER mRNA species in normal breast tissue compared to tumor tissue is an important issue and requires further study using more sensitive assay systems such as RNase protection and the polymerase chain reaction.

Two previous studies have also investigated the presence of altered sized ER mRNA. The study of Rio *et al.* (14) concluded there was no evidence for the presence of altered sized forms of estrogen receptor mRNA. The second study by Barrett-Lee *et al.* (13) observed the presence of a smaller 3.7 kb transcript which was present in higher abundance in tumors than in MCF 7 breast cancer cells. Our findings very clearly show the presence of this transcript as well as other sized transcripts. The discrepancies between our observations and the other two may reflect the fact that we have always used large amounts (10-20 μ g) of poly(A⁺) enriched RNA for our analyses. Rio *et al.* (14) have only used total RNA and Barrett-Lee *et al.* (13) have used either total RNA or very small amounts (2.5 μ g) of poly(A⁺) RNA. Another possible reason for the

differences may be our selection of tumors which was confined to only large tumors, and this may have biased our results.

MATERIALS AND METHODS

Materials

³²P-dCTP (3000 Ci/mmol) was purchased from New England Nuclear (Lachaire, Quebec). Restriction enzymes, T₄ DNA ligase and vectors used for subcloning (pGEM) were from Promega (Bio/Can Scientific Inc., Mississauga, Ontario). All other molecular biology grade reagents were from Fisher Scientific (Winnipeg, Manitoba).

Human Breast Tumor and Cell Samples

Breast tumor biopsies sent frozen to Dr. H. G. Friesen's laboratory for routine estrogen and progesterone receptor assay were the source of breast tumor tissue. T-47D and MCF 7 human breast cancer cells were grown as previously described (24).

RNA Isolation and Northern Blot Analysis

RNA was isolated by the guanidinium thiocyanate-caesium chloride method (25). Poly(A)⁺ enriched RNA was isolated by one cycle of oligo(dT)-cellulose chromatography (26). It should be noted that this one cycle of oligo(dT)-cellulose chromatography was insufficient to remove all the ribosomal RNA (see Figs. 1C and 2B). We have therefore referred to the RNA used in these studies as poly(A)⁺ enriched RNA. Ten to 20 µg poly(A)⁺ were denatured in 50% (vol/vol) formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1% (wt/vol) agarose gels containing 2.2 M formaldehyde and then blotted onto nitrocellulose (27). Filters were baked for 2 h at 80 °C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized with the 2.1 kbp human ER cDNA, OR-8 insert (kindly provided by Dr. Pierre Chambon, (12)) labeled with ³²P by nick translation (Amersham, Oakville, Ontario) to a specific activity of 10⁸ cpm/µg (3 × 10⁷ cpm were used per hybridization). Hybridizations, usually for 48 h, were performed at 42 °C in the presence of 50% (vol/vol) deionized formamide, 5× Denhardt's solution (1× Denhardt's = 0.02% wt/vol each of BSA, Ficoll, and polyvinylpyrrolidone), 5× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA), 250 µg/ml denatured salmon sperm DNA and 0.1% sodium dodecyl sulfate (SDS). At the end of the hybridization period the blots were washed twice in 2× SSC, 0.1% SDS (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 15–30 min at room temperature, followed by three 20-min washes in 0.1× SSC, 0.1% SDS at 65 °C. After the signal had been allowed to decay the filters were hybridized sequentially with the subcloned fragments of OR-8, fragment O and then fragment J. Once again when the previous signal had decayed the filters were also hybridized with the 1.9 kbp human EGF cDNA insert from lambda EGF15(c) (28). Filters were exposed to Kodak XAR film at –70 °C with an intensifying screen.

DNA Isolation and Southern Blot Analysis

Genomic DNA, isolated from various human breast tumors was digested with *PvuII* or *PstI*, fractionated on a 0.8% alkaline-agarose gel and transferred to nitrocellulose according to the protocol of Davis *et al.* (29). Hybridization and washing procedures were essentially as described above.

ER Binding Analysis

Samples weighing between 0.2–0.3 g were taken for ER assay. Available receptors were assayed using a single saturating dose and a charcoal-dextran method (3) for separation of bound from free hormone. Receptor concentration was expressed as femtomoles of steroid bound per mg cytosol protein.

Acknowledgments

We would like to thank Dr. G. I. Bell for providing the human EGF cDNA and Dr. P. Chambon for providing the human estrogen receptor cDNA (OR8). We thank Dr. H. G. Friesen for providing the human breast tumor biopsy samples and Ingrid Pfeiffer for carrying out the cytosolic estrogen receptor assays. We thank John Karvelas for his assistance in preparation of RNA from some of the tumors.

Received December 22, 1988. Revision received January 20, 1989. Accepted January 20, 1989.

Address requests for reprints to: Leigh C. Murphy, Department of Biochemistry, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3.

This work was supported by the National Cancer Institute (Canada).

* NCI Scientist.

REFERENCES

1. Vorherr H (ed.) 1980 Breast Cancer. Urban & Schwarzenberg, Baltimore
2. Sledge GW, McGuire WL 1983 Steroid hormone receptors in human breast cancer. *Adv Cancer Res* 38:61–75
3. Murphy LC, Sutherland RL 1981 A high affinity binding site for the antioestrogens, tamoxifen and CI 628, in immature rat uterine cytosol, which is distinct from the oestrogen receptor. *J Endocrinol* 91:155–161
4. Sutherland RL, Murphy LC 1982 Mechanisms of oestrogen antagonism by nonsteroidal antioestrogens. *Mol Cell Endocrinol* 25:5–23
5. Maass H, Jonat W, Stolzenbach G, Trams G 1980 The problem of nonresponding estrogen receptor-positive patients with advanced breast cancer. *Cancer* 46:2835–2837
6. Sedlacek S, Horwitz K 1984 The role of progestins and progesterone receptors in the treatment of breast cancer. *Steroids* 44:467–484
7. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM 1986 The c-erb-A gene encodes a thyroid hormone receptor. *Nature* 324:641–646
8. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstromm B 1986 The c-erb-A protein is a high affinity receptor for thyroid hormone. *Nature* 324:635–640.
9. Godowski PJ, Rusconi S, Miesfield R, Yamamoto KR 1987 Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* 325:365–368
10. Hollenberg SM, Giguere V, Segui P, Evans RM 1987 Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. *Cell* 49:39–48
11. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P 1987 Functional domains of the human estrogen receptor. *Cell* 51:941–951
12. Green S, Walter P, Kumar V, Kurst A, Bonnet JM, Argos P, Chambon P 1986 Human oestrogen receptor cDNA:

- sequence, expression and homology to v-erb-A. *Nature* 320:134-139.
13. Barrett-Lee PJ, Travers MT, McClelland RA, Luqmani Y, Coombes RC 1987 Characterization of estrogen receptor messenger RNA in human breast cancer. *Cancer Res* 47:6653-6659
 - 13a. Murphy LC, Dotzlaw H, Regulation of transforming growth factor α (TGF α) and TGF β mRNA abundance in T-47D, human breast cancer cells. *Mol Endocrinol*, in press
 14. Rio MC, Bellocq JP, Gairard B, Rasmussen UB, Krust A, Koehl C, Calderoli H, Schiff V, Renaud R, Chambon P 1987 Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. *Proc Natl Acad Sci USA* 84:9243-9247
 15. Hill SM, Fuqua SAW, Charnness GC, Greene GL, McGuire WL 1989 Estrogen receptor expression in human breast cancer associated with an estrogen receptor gene restriction fragment length polymorphism. *Cancer Res* 49:145-148
 16. Yamamoto KR, Gehring U, Stampfer MR, Sibley C 1976 Genetic approaches to steroid hormone action. *Recent Prog Horm Res* 32:3-32
 17. Miesfield R, Okret S, Wikstrom A-C, Wrangé O, Gustafsson J-A, Yamamoto KR 1984 Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. *Nature* 312:779-781
 18. Hughes MR, Malloy PJ, Kieback DG, Kesterson RA, Pike JW, Feldman D, O'Malley BW 1988 Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science* 242:1702-1705
 19. Garcia T, Lehrer S, Bloomer WD, Schachter B 1988 A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors. *Mol Endocrinol* 2:785-791
 20. Berkenstam A, Glaumann H, Martin M, Gustafsson J-A, Norstedt G, Hormonal regulation of estrogen receptor mRNA in T-47D_{co} and MCF 7 cells. Program of the 2nd International CBT Symposium on the steroid/thyroid hormone receptor family and gene regulation. Stockholm, Sweden, 1988, p 72 (Abstract)
 21. Horwitz KB, Mockus MB, Lessay BA 1982 Variant T-47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell* 28:633-642
 22. Lin CR, Chen WS, Kruijer W, Stolarsky LS, Wber W, Evans RM, Verma IM, Gill GN, Rosenfield MG 1984 Expression cloning of the human EGF receptor cDNA: gene amplification and three related messenger RNA products in A 431 cells. *Science* 224:843-848
 23. Walter P, Green S, Grene G, Kurst A, Bomert J-M, Jeltsch J-M, Staub A, Jensen E, Scrace G, Waterfield M, Chambon P 1985 Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci USA* 82:7889-7893
 24. Murphy LC, Dotzlaw H 1989 Endogenous growth factor expression in T-47D, human breast cancer cells, associated with reduced sensitivity to the antiproliferative effects of progestins and antiestrogens. *Cancer Res* 49:599-604
 25. Chirgwin J, Fryzbyla A, MacDonald R, Rutter W 1979 Isolation of biologically active ribonucleic acid from sources enriched for ribonuclease. *Biochemistry* 18:5294-5298
 26. Aviv H, Leder P 1972 Purification of biologically active growth messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc Natl Acad Sci USA* 69:1408-1412
 27. Thomas PS 1980 Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 77:5201-5205
 28. Bell GI, Fong NM, Stempien MM, Wormsted MA, Caput D, Lalling K, Urdea MS, Rall LB, Sanchez-Pescador R 1986 Human epidermal growth factor precursor. cDNA sequence, expression *in vitro* and gene organization. *Nucleic Acids Res* 14:8427-8446
 29. Davis GL, Dibner MD, Battey JF 1986 Basic Methods in Molecular biology. Elsevier Sciences Publishing Co Inc, New York





IDENTIFICATION OF AN EXON 3 DELETION SPLICE VARIANT ANDROGEN RECEPTOR mRNA IN HUMAN BREAST CANCER

Xiang ZHU¹, Angela A.I. DAFFADA¹, Christina M.W. CHAN¹ and Mitchell DOWSETT^{1*}

¹Academic Department of Biochemistry, Royal Marsden Hospital, London, SW3 6JJ, UK

Androgens and androgen receptor (AR) are involved in many regulatory processes in the growth of female breast cells. Mutations in the AR gene and/or alterations of the AR protein sequence may be related to the development and progression of breast cancer. Using reverse transcription-polymerase chain reaction we have examined 31 female breast-cancer samples, 5 normal female breast tissues and 6 breast-cancer cell lines for the presence of splice variants of AR mRNA and have identified an exon 3 deletion splice variant ($\Delta 3$ AR). The higher expression of the variant relative to the wild-type AR (WT AR) was found in 7 breast-cancer samples ($\Delta 3$ /WT > 15%) and relatively lower levels of the variant were observed in 3 breast-cancer cell lines ($\Delta 3$ /WT < 5%). However, in normal breast tissues, expression of the variant was undetectable by Southern blot analysis. *In vitro* translation of the $\Delta 3$ AR mRNA resulted in a variant AR protein of about 105 kDa, smaller than the WT AR by about 5 kDa. We thus report an exon deletion splice variant of AR mRNA in breast cancer. The variant protein is predicted to lack the second zinc finger within the DNA-binding domain and is expected to be unable or to have reduced ability to bind to androgen-response elements and to activate transcription. The relatively high expression of this AR variant in some breast-cancer tissues may indicate its role in regulating the growth of these cancers. *Int. J. Cancer* 72:574–580, 1997.

© 1997 Wiley-Liss, Inc.

Androgens and androgen receptor (AR) may play an important role in the development and progression of female breast cancer. AR is the sex hormone receptor most frequently found in both primary and secondary breast tumours: between 76% and 91% are reported to be AR-positive, higher than oestrogen receptor (ER) (71–82%) and progesterone receptor (PR) (61–72%) (Kuenen-Boumeester *et al.*, 1992; Lea *et al.*, 1989; Soreide *et al.*, 1992). Although AR is expressed predominantly in ER-positive and PR-positive breast-cancer cell lines and tissues (Kuenen-Boumeester *et al.*, 1992), between 9% and 25% of breast cancers are reported to express AR as their sole sex hormone receptor (Kuenen-Boumeester *et al.*, 1992; Lea *et al.*, 1989), especially those of ductal carcinomas which have a relatively poor prognosis (Kuenen-Boumeester *et al.*, 1996). This high expression of AR, along with high levels of androgens in breast-cancer tissues (Bhatavdekar, 1987; Recchione *et al.*, 1995), may indicate the importance of AR in regulating the growth of breast-cancer cells. AR also may be important in hormonal therapy of breast cancer. Although not widely used nowadays, androgens have been useful for the treatment of patients who have poor responsiveness to oestrogen therapy (Cooperative Breast Cancer Group, 1964; Manni *et al.*, 1981) and the combined use of androgen and anti-oestrogen therapy has therapeutic advantages over anti-oestrogen treatment alone (Tormey *et al.*, 1983; Ingle *et al.*, 1991). Of more contemporary importance, the positive response to progestins (megestrol acetate or medroxyprogesterone acetate) in advanced breast-cancer patients has been determined by the AR level (Teulings *et al.*, 1980; Birrell *et al.*, 1995), and the progestin medroxyprogesterone acetate has been shown to inhibit proliferation of an ER- and PR-negative breast-cancer cell line *via* AR (Hackenberg *et al.*, 1993a).

AR is a member of the steroid/thyroid/retinoic acid receptor superfamily. Like all other members of this family, AR contains several functional domains which are encoded by 8 exons: the large N-terminal domain is encoded by exon 1, the DNA-binding domain consists of 2 zinc fingers which are separately encoded by exons 2 and 3 and the information for the ligand-binding domain is

distributed over exons 4–8 (Chang *et al.*, 1988; Trapman *et al.*, 1988). In the absence of androgens, AR exists in a non-DNA-binding state as an inactive complex with heat shock proteins. Upon binding of androgens, AR dissociates from this complex, reveals the DNA-binding domain, dimerises, binds to the androgen-responsive element (ARE) of the regulated gene with 2 zinc fingers and thereby activates specific gene transcription (Carson-Jurica *et al.*, 1990).

Extensive studies have been performed on structural alterations of ER in breast cancer. There is now substantial evidence for a mixture of wild-type (WT) and variant ER mRNAs in breast-cancer cell lines and tissues (Murphy and Dotzlaw 1989; McGuire *et al.*, 1992; Miksicek *et al.*, 1993). Some of the predicted variant ER proteins lack some functional domains, exhibit altered functions or interfere with WT ER function when expressed in transfected cells. For example, the exon 5 deletion variant ER acts as a dominant-positive regulator of WT ER which constitutively activates transcription in the absence of oestrogens (Fuqua *et al.*, 1991; Castles *et al.*, 1995), while exon 3 and exon 7 deleted variants behave as dominant-negative transcription factors which prohibit normal ER from binding to the oestrogen response element and from activating transcription (Wang and Miksicek, 1991; Fuqua *et al.*, 1992). Although it remains unclear whether the variant ER mRNAs are translated *in vivo*, a truncated exon 5-deleted ER protein has been detected in BT-20 breast-cancer cells (Castles *et al.*, 1995) and statistically significant relationships have been found between variant/WT mRNA levels and a number of clinical and biological variables (McGuire *et al.*, 1992; Daffada *et al.*, 1995). Some studies have led to speculation that ER variants may be involved in anti-oestrogen resistance and tumour progression (Fuqua, 1994; Daffada *et al.*, 1995; Lemieux and Fuqua, 1996). For example, the relative levels of certain variant ER mRNAs increased with breast-tumour progression (Fuqua *et al.*, 1991; McGuire *et al.*, 1992; Murphy *et al.*, 1995). However, a report on the presence of several ER variants in a normal breast sample makes it unlikely that splicing variants are an indicator of malignancy (Pfeffer *et al.*, 1995). Another report comparing the expression of ER variants in 9 normal and 19 neoplastic breast samples has shown that expression levels of exon 5 deletion variant ER mRNA relative to WT ER mRNA were significantly higher in neoplastic than in normal breast tissues (Leygue *et al.*, 1996). It has been speculated, therefore, that the generation of specific ER variants may be deregulated in breast-cancer tissues and that such deregulation may contribute to the progression of breast cancer (Leygue *et al.*, 1996).

In contrast to ER, in which a large numbers of splice variants have been described, AR has been relatively poorly studied in breast cancer. No AR splice variant has been reported so far in

Contract grant sponsor: European Commission International Scientific Cooperation Programme Marie Curie Fellowship; contract grant number: ERBCIST 940200.

*Correspondence to: Academic Department of Biochemistry, Royal Marsden Hospital, Fulham Road, London, SW3 6JJ, UK. Fax: 44-171-376-3918. E-mail: m.dowsett@icr.ac.uk

Received 13 November 1996; revised 26 March 1997

breast cancer. Considering the parallel between ER and AR, we have hypothesised that the same events could also occur in AR mRNA and that alteration of the AR sequence could lead to a change in androgen action within breast cancer cells. Therefore, we have investigated AR transcripts in a number of normal and tumour breast tissues and cell lines. Using reverse-transcription (RT)-PCR we screened for variations in the DNA-binding domain (exons 2 and 3) and the ligand-binding domain (exons 4–8). We have identified a variant AR mRNA lacking exon 3 in some breast-cancer samples and cell lines. Expression levels of the variant relative to the WT message varied considerably between samples. The present report describes the isolation, sequencing and *in vitro* translation of the variant transcript.

MATERIAL AND METHODS

Cell lines and tissues

Breast-cancer cell lines T47D, ZR-75-1 and BT-20 were grown in IMEM medium (GIBCO, Paisley, UK) containing 5% FCS (GIBCO); MCF-7, BT-474 and MDA-MB-231 were grown in RPMI 1640 medium (GIBCO) containing 10% FBS. All of the media contained 2 mM L-glutamine, 5 U/ml penicillin, 5 mg/ml streptomycin and 12.5 ng/ml amphotericin B (Sigma, Poole, UK). Upon reaching 80% confluence, cells were collected by EDTA/trypsin treatment and snap-frozen in liquid nitrogen until they were used for RNA extraction. Five normal female breast samples were collected from patients undergoing reduction mammoplasty and stored at -70°C until analysed. Tissues were confirmed to be normal by histopathologic analysis. Thirty-one breast-cancer samples were obtained through the breast tumour bank at Royal Marsden Hospital, London, UK. The diagnosis was made morphologically by the Department of Pathology of the hospital. Frozen tissues were pulverised in a dismembrator (Braun Biotech, Aylesbury, UK) prior to RNA extraction.

Extraction of total RNA

Total RNA was extracted from the frozen cells and pulverised tissues using RNeasy B (Qiagen, Crawley, UK) as recommended by the manufacturer. RNA concentrations were determined spectrophotometrically.

RT-PCR

Total RNA (2 μg) was denatured at 65°C for 3 min and reverse-transcribed in the presence of 1 mM dNTPs, 1 unit/ μl

RNAse inhibitor, 20 mM random primers (Promega, Southampton, UK), 5 mM dithiothreitol (GIBCO), 5 U/ μl reverse transcriptase and buffer (superscript; GIBCO) for 20 min at 23°C , 60 min at 42°C and 10 min at 95°C . PCR amplifications were performed with the use of reverse-transcription mixture in a final volume of 100 μl in the presence of 0.4 U Taq polymerase and buffer (Perkin Elmer, Beaconsfield, UK) and 500 nM of each primer. Five sets of primers, spanning exons 2–3, 5–6, 7–8, 5–8 and 2–4, respectively, were used in this study (Fig. 1). Each PCR consisted of 35 cycles (1 min at 95°C , 1 min at 55°C and 1 min at 72°C) using a DNA thermal cycler (Perkin Elmer). PCR products were electrophoresed on 2% ethidium bromide-stained agarose gels and photographed under UV illumination. Two RT-PCR amplifications were performed to confirm the similarity of the results.

Southern blot analysis

PCR products (10 μl) were loaded onto 2% agarose gels and electrophoresed for 2 hr at 80 V prior to denaturation of DNA in 0.6 M NaCl, 0.2 M NaOH (45 min) and neutralisation in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4) (30 min) with gentle agitation. DNAs were transferred onto nylon membranes using $20 \times \text{SSC}$ (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and UV cross-linked (Stratagene, Cambridge, UK). Pre-hybridisation and hybridisation (2 and 18 hr, respectively) were carried out in 7% SDS, 10 mM sodium phosphate buffer (pH 7.3) and 1 mM EDTA at 42°C in a Techne (Cambridge, UK) hybridisation oven. The exon 2-specific probe (AAC ATG GTC CCT GGC AGT CTC, bases 2146–2166), exon 3-specific probe (CAG AAG TAC CTG TGC GCC AGC, bases 2299–2319) or exon 4-specific probe (ACT GAG GAG ACA ACC CAG AAG, bases 2482–2502) was end-labelled with [^{32}P]dATP (DuPont, Stevenage, UK) using T4 polynucleotide kinase (GIBCO) as recommended by the manufacturer. Stringency washes (3–5 washes of 5 min at 42°C – 50°C) were performed in 5% SDS, 10 mM sodium phosphate buffer (pH 7.3) and 1 mM EDTA. Membranes were then exposed to X-ray film at -70°C with intensifying screens to visualise the results. Quantification of WT and variant bands was performed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) as reported previously (Daffada *et al.*, 1995), and a % $\Delta 3/\text{WT}$ value was calculated.

Restriction analysis of PCR products

PCR products (10 μl) amplified by primers 1 and 2 from a breast-tumour sample (T3) were incubated with 10 U restriction

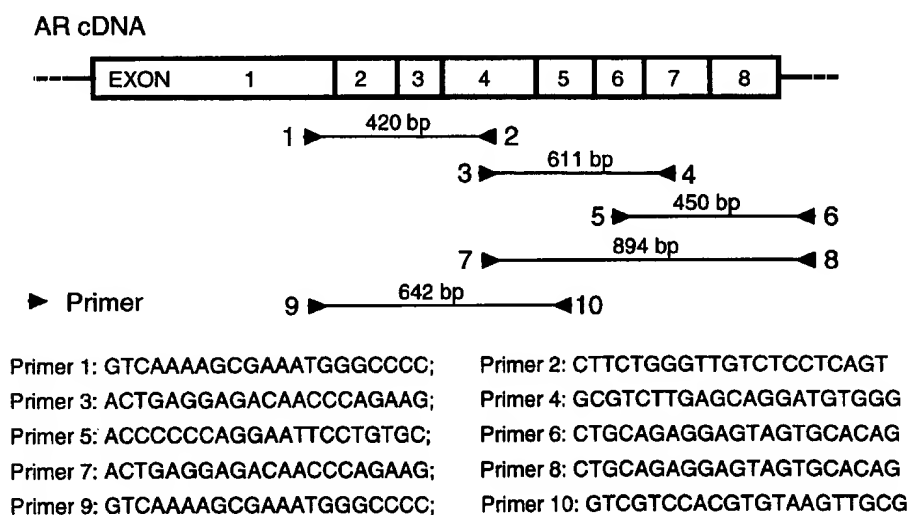


FIGURE 1 – Schematic representation of WT androgen receptor cDNA and the positions of primers used to detect exon deletion AR variants. The sequences of the primers also are shown (AR sequence: Chang *et al.*, 1988).

enzyme Acs I (Boehringer Mannheim, Lewes, UK) at 50°C for 2 hr. After incubation, the digested products along with the undigested products were subjected to electrophoresis on a 2% ethidium bromide-stained agarose gel and photographed.

Cloning and sequencing of PCR products

PCR products (50 µl) were precipitated using sodium acetate and ethanol for more than 2 hr at -20°C and centrifuged at 14,000 g for 20 min at 4°C. The pellet was washed with 100 µl of 70% ethanol,

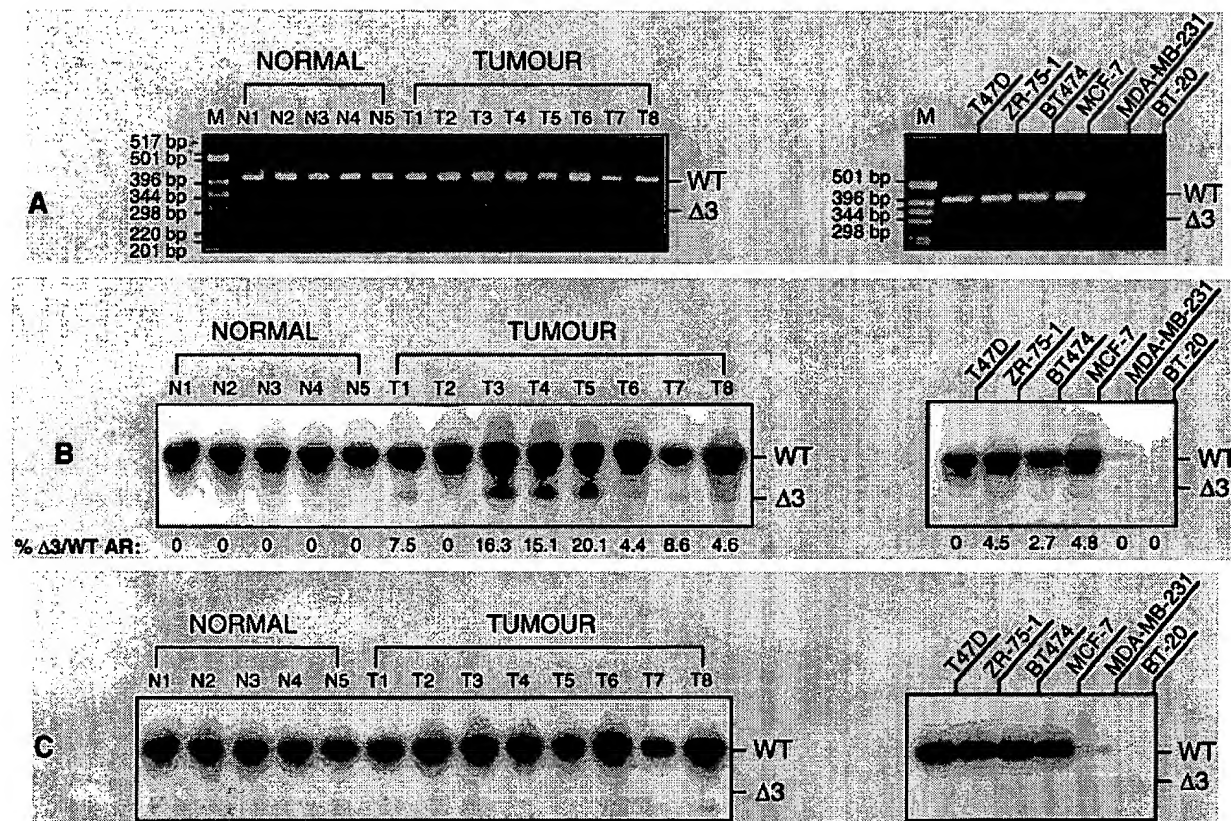


FIGURE 2 – Two micrograms of RNA from 5 normal breast tissues (lanes N1–N5), 8 breast-tumour samples (lanes T1–T8) (left side) and 6 breast-cancer cell lines (right side) were subjected to RT-PCR using primers 1 and 2, which amplified across exons 2 and 3 of the human AR sequence. PCR products were electrophoresed (a), blotted onto nylon membranes and hybridised with ³²P-labelled exon 2-specific probe (b) and exon 3-specific probe (c). Results were viewed after autoradiography. Quantitation of the WT and Δ3 bands was by phosphorimaging, and percentages of Δ3/WT AR ratios were calculated (bottom of b).

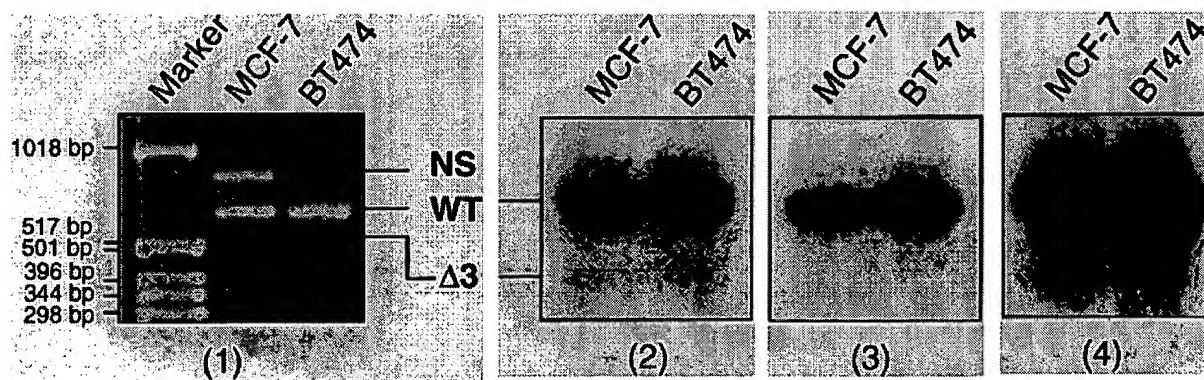


FIGURE 3 – RT-PCR amplification across exons 2, 3 and 4 of human AR sequence in MCF-7 and BT-474 breast-cancer cell lines using primers 9 and 10 (Fig. 1). The products visualised by electrophoresis (1) showed a wild-type (WT) band and a variant (Δ3) band. Southern blot hybridisation with the exon 2-specific probe (2) and the exon 4-specific probe (4) showed both WT and variant bands, while with the exon 3-specific probe the variant band was absent (3). NS, non-specific.

air-dried briefly and resuspended in 10 µl water. Concentrated cDNA was then run on a 2% NuSieve gel (Flowgen, Sittingbourne, UK). The WT and variant bands were sliced and extracted from the gel and cloned into pCR II vectors, which were then transformed into *Escherichia coli* cells (TA cloning kit; Invitrogen, Abingdon, UK) as suggested by the manufacturer. Positive clones were selected and amplified by culturing of the transformed cells in 5 ml LB/ampicillin broth. Plasmid DNAs were then prepared from the cells, digested with Eco RI restriction enzyme, run on a 1% agarose gel and blotted onto a nylon membrane. Clones containing WT and variant AR sequences were selected by Southern blot hybridisation with exon 2- and exon 3-specific probes. Recombinant plasmid DNAs were purified using a plasmid purification kit (Qiagen, Dorking, UK). Sequencing was then carried out using the Circum-Vent Thermal Cycle Sequencing kit (New England Biolabs, Hitchin, UK).

In vitro translation of the variant receptor protein

A Hind III/Bst EII AR fragment (1505 bp) and a Bst EII/Bam HI AR fragment (1,563 bp) were cleaved from AR expression vector pSVARo (kindly provided by Dr. Brinkmann, Erasmus University, Rotterdam, The Netherlands), gel-purified and ligated to form a Hind III/Bam HI fragment which contains the whole protein-coding region of AR. This Hind III/Bam HI AR fragment was then ligated into the corresponding sites of the pSP64 vector to generate the WT AR expression vector (pSP64-WTAR). The variant AR expression vector (pSP64-Δ3AR) was the same as the WT vector except that a Bst EII/Tth111 I fragment in the AR cDNA was replaced by an exon 3-deleted Bst EII/Tth111 I fragment, which was derived from the PCR product. The correct insertion of WT and Δ3 AR fragments in pSP64 vectors was confirmed by restriction analysis and sequencing. *In vitro* transcription and translation reactions were performed using 1 µg pSP64-WTAR or pSP64-Δ3AR vectors as DNA templates in the TNT-coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. [³⁵S]-Methionine was incorporated during translation to allow visualisation of synthesised polypeptides by SDS-PAGE followed by autoradiography.

RESULTS

Total RNA extracted from breast-tissue samples and cell lines was reverse-transcribed and used as the template for PCR amplifications using 5 primer sets (Fig. 1). With the use of primers 1 and 2, 2 amplification products of different sizes were yielded in both tissue samples and cell lines (Fig. 2a). The slower migrating band corresponded well to the expected length of WT AR (420 bp), while the faster migrating band was of approx. 300 bp. To determine whether the slower migrating band corresponded to the sequence of AR and whether the unexpected faster migrating band was related to it, we carried out Southern blot and hybridisation analyses using 2 probes specific to exons 2 and 3, respectively. With the exon 2-specific probe, hybridisation to both slower and faster bands was observed (Fig. 2b), while using the exon 3-specific probe, hybridisation to the fast band was absent (Fig. 2c), indicating that the unexpected PCR product (faster band) resulted from a variant AR mRNA lacking at least a portion of exon 3. Another independent RT-PCR using a different combined primer set (primers 9 and 10) to amplify exons 2–4 in breast-cancer cell lines MCF-7 and BT474 also yielded 2 bands which corresponded to the predicted WT (642 bp) and exon 3 deletion (525 bp) AR fragments (Fig. 3, part 1). Although the variant band was weaker in the 2 cell lines, it clearly hybridised to exon 2- and exon 4-specific probes (Fig. 3, parts 2, 4) but not to the exon 3-specific probe (Fig. 3, part 3). Amplification of the Δ3 variant by 2 different PCRs makes it unlikely that the variant product was artefactual. The bands higher than WT in tumor samples T2 and T8 (Fig. 2a) and the MCF-7 cell line (Fig. 3, part 1) were considered to be non-specific because they were not detected by Southern blot analysis (Fig. 2b,

c; Fig. 3, parts 2–4). A slight band at a lower size than Δ3AR in samples N2–5 and T1–7 (Fig. 2a), which also was hybridised by the exon 3-specific probe (Fig. 2c) and the bands intermediate in size between WT and Δ3 in T47D cells and higher in size than WT in ZR-75-1 and MCF-7 cells (Fig. 2b), currently remains uncharacterised and may or may not be specific.

Restriction analysis of the PCR products of a breast-cancer sample (T3) was performed using restriction enzyme Acs I, which cleaves the sequence of ATCGT located in exon 3. Deletion of exon 3 removes this restriction site so that the variant fragment should not be digested. The restriction patterns of Acs I observed in WT and variant fragments were as expected: the WT fragment (420 bp) was digested into 2 smaller fragments (259 bp and 161 bp), whereas the variant fragment (303 bp) was not digested (Fig. 4). This result also suggested loss of the exon 3 sequence in the variant fragment.

Comparison of the sequence between variant and WT AR cDNA demonstrated that the sequence of variant AR was identical to that of WT AR in exons 2 and 4 but that the sequence corresponding to exon 3 was entirely absent (Fig. 5). Deletion of exon 3 corresponds precisely to known exon/intron boundaries, indicating that this variant is generated through alternative splicing of the primary AR mRNA transcript.

The relative expression levels of the Δ3AR measured by phosphorimaging varied greatly between different samples (Table 1). Relatively higher expression levels of the variant (*i.e.*, Δ3/WT > 15%) were found in 7 of 31 samples. Δ3AR was detected in ZR-75-1, BT-474 and MCF-7 cell lines, but the expression levels were relatively low (Δ3/WT < 5%). In 5 normal breast tissues, expression of the variant was undetectable by phosphorimaging analysis (Fig. 2b), though a slight band at the same level as Δ3AR was visible in most of the normal tissues (Fig. 2a).

In vitro translation experiments with [³⁵S]-methionine were performed to determine whether the Δ3AR mRNA could be produced faithfully in rabbit reticulocyte lysates. Figure 6 is an autoradiograph of the translation products analysed on SDS-PAGE. As predicted from the cDNA sequence, the WT AR polypeptide has

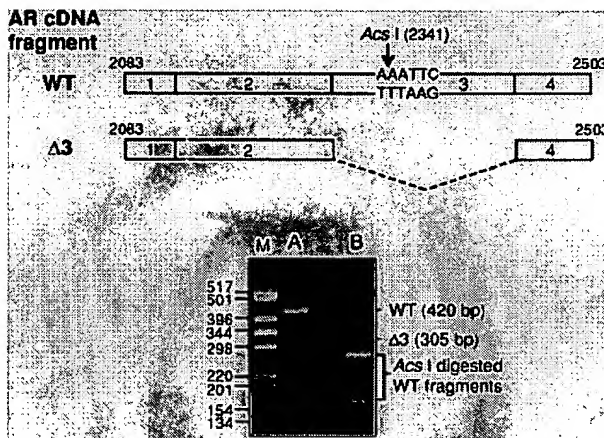


FIGURE 4 – Restriction analysis of RT-PCR products. Top: Diagram of WT AR and exon 3-deleted variant (Δ3) AR cDNA fragments amplified by RT-PCR. An Acs I restriction site (nt: 2341) exists in exon 3 of the WT AR fragment but not in the Δ3AR fragment. Numbering is according to Chang *et al.* (1988). Bottom: Gel electrophoresis of digested and undigested RT-PCR products. Lane M, DNA markers; lane A, undigested AR cDNA products showing both WT band (420 bp) and variant band (303 bp); lane B, the same AR cDNA products were digested with restriction enzyme Acs I at 50°C for 2 hr. The WT fragment was digested into 2 smaller fragments (259 and 161 bp), whereas the variant fragment was not digested.

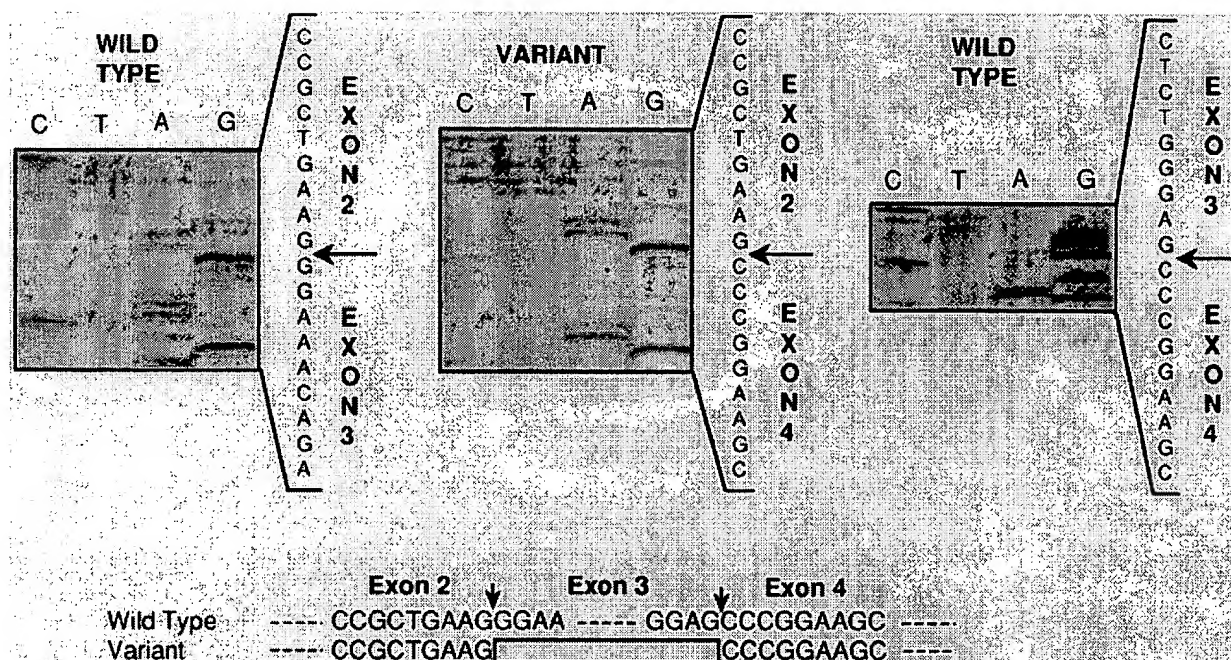


FIGURE 5 – Dideoxy sequence analysis of the variant AR mRNA missing exon 3. Arrows, exon boundaries.

TABLE 1 – RATIO OF $\Delta 3$ VARIANT AR TO WT AR IN BREAST TUMOURS, CELL LINES AND NORMAL BREAST TISSUES

Sample	Number	% $\Delta 3$ /WT					
		0	0-5	5-10	10-15	15-20	>20
Tumour	31	13	3	6	2	4	3
Cell lines	6	3	3				
Normal	5	5					

an m.w. of 110 kDa, whereas the $\Delta 3$ AR has mobility of 105 kDa due to the in-frame deletion of 39 amino acids.

DISCUSSION

The alternative splicing seen for ER does not appear to be a common feature of messages coding for other members of the steroid receptor superfamily. Attempts to find splicing variants in other steroid receptors have been reported as unsuccessful (Pfeffer *et al.*, 1995). The finding of the $\Delta 3$ AR variant is therefore significant in establishing that this is not unique to ER among sex steroid receptors. A further variant has been described for glucocorticoid receptor (Moalli *et al.*, 1993).

The expression of splice variants of steroid receptors appears to be not only receptor-specific but also tissue- and sample-specific. A $\Delta 4/7$ ER variant previously identified in our laboratory has been shown to be expressed in normal endometrial and breast-cancer samples but not in liver tissues (Daffada and Dowsett, 1995). The $\Delta 3$ ER variant has been found by different groups in the MCF-7 breast-cancer cell line and breast-cancer tissues (McGuire *et al.*, 1992; Miksicek *et al.*, 1993), but it could not be detected by Pfeffer *et al.* (1995) in their MCF-7 cell line and breast-cancer samples, though the authors believed that it could have escaped detection by 2 PCR amplifications with 2 different primer sets. We have observed that $\Delta 3$ AR was not detectable in some breast-cancer samples, though the WT AR product was as great as with the other samples which expressed the variant pattern. These tissue- and

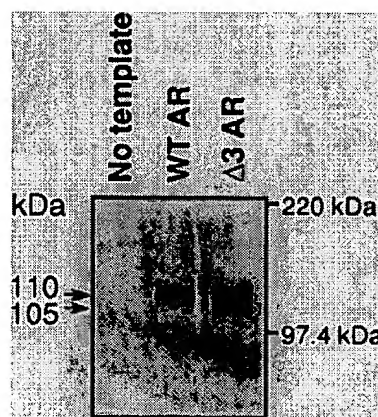


FIGURE 6 – *In vitro* translation of WT AR and exon 3-deleted variant androgen receptor ($\Delta 3$ AR) cDNAs. WT and $\Delta 3$ AR cDNAs were cloned into pSP64 vector (see "Material and Methods"). Plasmid DNAs were used for coupled *in vitro* transcription and translation, using a commercial kit (Promega). [35 S]Methionine was incorporated during translation to allow visualisation of the synthesised polypeptide by SDS-PAGE followed by autoradiography. Arrows indicate synthesised AR products. The positions of Rainbow m.w. markers are given in kDa on the right.

sample-specific expressions of splicing variants seem unlikely to be the by-product of an imprecise splicing process without functional significance (Pfeffer *et al.*, 1995) since the by-product would then be expected to occur without difference between cells and tissues that express the same receptor(s). Thus, it may be speculated that the mechanisms which generate exon(s) splicing variants may be regulated differently in various tissues and that these regulations may be important in physiological and/or pathological processes.

The probable functional activity of variant Δ 3AR protein may be summarised by consideration of the known functional domains of the WT AR. An intact DNA-binding domain is crucial for normal functioning of steroid receptors (Hollenberg and Evans, 1988; Green *et al.*, 1988). The second zinc finger encoded by exon 3 is important in orientating the receptor for DNA binding (Berg, 1989), in stabilising DNA-protein interaction (Green *et al.*, 1988; Berg, 1989) and in providing the interface for receptor dimerisation (Luisi *et al.*, 1991). Single-amino-acid aberrations in the second zinc finger of the DNA-binding domain of AR in androgen insensitivity syndrome (AIS) have been described in association with androgen resistance *in vivo*, and these reduced or diminished transcriptional activation when recreated *in vitro* (Marcelli *et al.*, 1990; Klocker *et al.*, 1992; Kaspar *et al.*, 1993; Beitel *et al.*, 1994). Partial deletion of the DNA-binding domain *in vitro* completely inactivates the ability of receptor protein to stimulate transcription, though normal steroid-binding properties are retained (Hollenberg *et al.*, 1987). Deletion of exon 3 in the AR gene has been reported in AIS (Quigley *et al.*, 1992). The resultant protein had markedly reduced DNA-binding affinity and failed to activate transcription of an androgen-responsive reporter gene (Quigley *et al.*, 1992). Analogous studies of the exon 3 splice variant of ER (Wang and Miksicek, 1991; Miksicek *et al.*, 1993) also have demonstrated that this variant ER is unable to bind to oestrogen response element (ERE) *in vitro* or to activate transcription of a reporter gene containing an ERE *in vivo*. However, it has been shown to inhibit oestrogen-dependent transcription activation in a dominant-negative fashion when it is co-transfected with the WT ER and reporter plasmid. It also inhibits DNA binding of WT ER in a gel mobility shift assay *in vitro*. The function of the Δ 3AR protein in breast cancer remains to be explored. The absence of 53% (36 of 68 amino acids, AR sequence according to Chang *et al.*, 1988) of the DNA-binding domain in the putative variant receptor is likely to prevent it from binding to its target gene, the ARE, and to activate gene transcription.

Predictions of the possible role of the Δ 3AR variant protein, if it exists, in the development and progression of breast cancer must be made largely on the basis of the above functional considerations. It is well known that the growth of breast cells is regulated by a complex interplay of different steroid hormones, of which androgens and oestrogens are among the most closely related pair due to their related metabolic pathways (Dauvois and Labrie, 1990). As mentioned above, the presence of Δ 3AR may reduce or abolish the

DNA-binding capacity of AR. Thus, the growth-inhibitory role of androgens through AR may be reduced or lost. However, the metabolites of androgens (such as androst-5-ene-3 β , 17 β -diol and 5 α -androstane-3 β , 17 β -diol, which have oestrogenic properties) along with oestrogens may continue to have a growth-stimulatory effect through interaction with ER (Hackenberg *et al.*, 1993b; Boccuzzi *et al.*, 1994). Thus, expression or possibly over-expression of Δ 3AR may result in a reduced inhibitory action of androgens within breast cells and, therefore, might favour the cell proliferation which is involved in the development and progression of breast cancer. The observation that point mutations in exon 3 of the AR gene existed in 3 male breast-cancer patients with androgen resistance (Wooster *et al.*, 1992; Lobaccaro *et al.*, 1993) supports the concept that altered AR protein may be of biological and clinical importance in breast cancer.

However, the clinical significance of exon(s) splicing in steroid receptors remains uncertain. There are very few data which confirm the existence of the translated protein product of these mRNA variants in untransfected cells. Thus, while functional changes to receptors have been described after transfection, their physiological and pathological significance remains unclear.

In conclusion, we have identified an exon 3-deleted splice variant AR mRNA in female breast-cancer samples and breast-cancer cell lines. Unlike ER, this is the only AR splice variant that has been described in breast cancer. Moreover, by comparison of the expression levels of the variant relative to WT, we have shown that there was relatively high expression of the variant in some breast-cancer samples in comparison to other samples and normal breast tissues. Our data suggest that the mechanisms generating exon deletion splice variants are not restricted to ER in breast cancer and that expression of various splicing variants in different receptors and different tissues may be controlled by gene regulation. The relatively high expression of the Δ 3AR variant in some breast cancers indicates a role for it in regulating the growth of these cancers. However, the ultimate significance of the variant AR remains to be determined by studies of the functionality of variant protein in breast tissues.

ACKNOWLEDGEMENTS

This study was supported partly by the Marie Curie Fellowship (application ERBCIST 940200), which is part of the European Commission International Scientific Cooperation Programme.

REFERENCES

- BEITEL, L.K., PRIOR, L., VASILIOU, D.M., GOTTLIEB, B., KAUFMAN, M., LUMBROSO, R., ALVARADO, C., MCGILLIVRAY, B., TRIFIRO, M. and PINSKY, L., Complete androgen insensitivity due to mutations in the probable α -helical segments of the DNA-binding domain in the human androgen receptor. *Hum. mol. Genet.*, **3**, 21–27 (1994).
- BERG, J., DNA binding specificity of steroid receptors. *Cell*, **57**, 1065–1068 (1989).
- BHATAVDEKAR, J.M., Peptide and steroid hormone levels in pre- and postmenopausal breast carcinoma. *Neoplasma*, **34**, 95–99 (1987).
- BIRRELL, S.N., RODER, D.M., HORSFALL, D.J., BENTEL, J.M. and TILLEY, W.D., Medroxyprogesterone acetate therapy in advanced breast cancer: the predictive value of androgen receptor expression. *J. clin. Oncol.*, **31**, 1572–1577 (1995).
- BOCCUZZI, G., BRIGNARDELLO, E., DI MONACO, M., GATTO, V., LEONARDI, L., PIZZINI, A. and GALLO, M., 5-En-androstene-3 beta, 17 beta-diol inhibits the growth of MCF-7 breast cancer cells when oestrogen receptors are blocked by oestradiol. *Brit. J. Cancer*, **70**, 1035–1039 (1994).
- CARSON-JURICA, M.A., SCHRADER, W.T. and O'MALLEY, B., Steroid receptor family: structure and functions. *Endocrinol. Rev.*, **11**, 201–220 (1990).
- CASTLES, C.G., FUQUA, S.A.W., KLOTZ, D.M. and HILL, S.M., Expression of a constitutively active estrogen receptor variant in the estrogen-negative BT-20 breast cancer cell line. *Cancer Res.*, **53**, 5934–5939 (1995).
- CHANG, C.S., KOKONTIS, J. and LIAO, S.T., Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. nat. Acad. Sci. (Wash.)*, **85**, 7211–7215 (1988).
- COOPERATIVE BREAST CANCER GROUP, Testosterone propionate therapy in breast cancer. *J. amer. med. Ass.*, **188**, 1069 (1964).
- DAFFADA, A.A.I. and DOWSETT, M., Tissue-dependent expression of a novel splice variant of the human oestrogen receptor. *J. Steroid Biochem. mol. Biol.*, **55**, 413–421 (1995).
- DAFFADA, A.A.I., JOHNSTON, S.R.D., SMITH, I.E., DETRE, S., KING, N. and DOWSETT, M., Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. *Cancer Res.*, **55**, 288–293 (1995).
- DAUVOIS, S., LI, S. and LABRIE, F., Antagonism between androgens and estrogens on the growth of mammary carcinoma *in vivo*. *Ann. N.Y. Acad. Sci.*, **595**, 413–415 (1990).
- FUQUA, S.A.W., Estrogen receptor mutagenesis and hormone resistance. *Cancer*, **74**, 1026–1029 (1994).
- FUQUA, S.A.W., FITZGERALD, S.D., ALLRED, D.C., ELLEDGE, R.M., NAWAZ, Z., McDONNELL, D.P., O'MALLEY, B.W., GREENE, G.L. and MCGUIRE, W.L., Inhibition of estrogen receptor activity by a naturally occurring variant in human breast tumors. *Cancer Res.*, **52**, 483–486 (1992).
- FUQUA, S.A.W., FITZGERALD, S.D., CHAMNESS, G.C., TANDON, A.K., McDONNELL, D., NAWAZ, Z., O'MALLEY, B.W. and MCGUIRE, W.L., Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.*, **51**, 105–109 (1991).

- GREEN, S., KUMAR, V., THEULAZ, I., WAHLI, W. and CHAMBON, P., The N-terminal DNA-binding "zinc finger" of the oestrogen and glucocorticoid receptors determines target gene specificity. *EMBO J.*, **7**, 3037-3044 (1988).
- HACKENBERG, R., HAWIGHORST, T., FILMER, A., NIA, A.H. and SCHULZ, K.D., Medroxyprogesterone acetate inhibits the proliferation of estrogen- and progesterone-receptor negative MFM-223 human mammary cancer cells via the androgen receptor. *Breast Cancer Res. Treat.*, **25**, 217-224 (1993a).
- HACKENBERG, R., TURGETTO, I., FILMER, A. and SCHULZ, K.D., Estrogen and androgen receptor mediated stimulation and inhibition of proliferation by androst-5-ene-3 beta, 17 beta-diol in human mammary cancer cells. *J. Steroid Biochem. mol. Biol.*, **46**, 597-603 (1993b).
- HOLLENBERG, S.M. and EVANS, R.M., Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell*, **55**, 899-906 (1988).
- HOLLENBERG, S.M., GIGUÈRE, V., SEGUI, P. and EVANS, R.M., Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. *Cell*, **49**, 39-46 (1987).
- INGLE, J.N., TWITO, D.I., SCHAID, D.J., CULLINAN, S.A., KROOK, J.E., MAILLIARD, J.A., TSCHETTER, L.K., LONG, H.J., GERSTNER, J.G., WINDSCHIT, H.E., LEVITT, R. and PFEIFLE, D.M., Combination hormonal therapy with tamoxifen plus fluoxymesterone versus tamoxifen alone in postmenopausal women with metastatic breast cancer. *Cancer*, **67**, 886-891 (1991).
- KASPAR, F., KLOCKER, H., DENNINGER, A. and CATO, A.C.B., A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors. *Mol. cell. Biol.*, **13**, 7850-7858 (1993).
- KLOCKER, H., KASPAR, F., EBERLE, J., ÜBERREITER, S., RADMAYR, C. and BARTSCH, G., Point mutation in the DNA binding domain of the androgen receptor in two families with Reifenstein syndrome. *Amer. J. hum. Genet.*, **50**, 1318-1327 (1992).
- KUENEN-BOUMEESTER, V., VAN DER KWAST, T.H., CLAASSEN, C.C., LOOK, M.P., LIEM, G.S., KLUN, J.G.M. and HENZEN-LOGMANS, S.C., The clinical significance of androgen receptors in breast cancer and their relation to histological and cell biological parameters. *Europ. J. Cancer*, **32A**, 1560-1565 (1996).
- KUENEN-BOUMEESTER, V., VAN DER KWAST, T.H., VAN PUTTEN, W.L.J., CLAASSEN, C., VAN OOUEN, B. and HENZEN-LOGMANS, S.C., Immunohistochemical determination of androgen receptors in relation to oestrogen and progesterone receptors in female breast cancer. *Int. J. Cancer*, **52**, 581-584 (1992).
- LEA, O.A., KVINNSLAND, S. and THORSEN, T., Improved measurement of androgen receptors in human breast cancer. *Cancer Res.*, **49**, 7162-7167 (1989).
- LEMIEUX, P. and FUQUA, S., The role of the estrogen receptor in tumor progression. *J. Steroid Biochem. Biol.*, **56**, 87-91 (1996).
- LEYGUE, E.R., WATSON, P.H. and MURPHY, L.C., Estrogen receptor variants in normal human mammary tissue. *J. nat. Cancer Inst.*, **88**, 284-289 (1996).
- LOBACCARO, J.M., LUMBROSO, S., BELON, C., GALTIER, D.F., BRINGER, J., LESIMPLE, T., NAMER, M., CUTULI, B.F., PUJOL, H. and SULTAN, C., Androgen receptor gene mutation in male breast cancer. *Hum. mol. Genet.*, **2**, 1799-802 (1993).
- LUISI, B.F., XU, W.X., OTWINOWSKI, Z., FREEDMAN, L.P., YAMAMOTO, K.R. and SIGLER, P.B., Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature (Lond.)*, **352**, 497-505 (1991).
- MANNI, A., ARAFAH, B. and PEARSON, O., Androgen-induced remissions after antiestrogen and hypophysectomy in stage IV breast cancer. *Cancer*, **48**, 2507-2509 (1981).
- MARCELLI, M., TILLEY, W.D., WILSON, C.M., GRIFFIN, J.E., WILSON, J.D. and MCPHAUL, M.J., Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. *Mol. Endocrinol.*, **4**, 1105-1116 (1990).
- MCGUIRE, W.L., CHAMNESS, G.C. and FUQUA, S.A., Abnormal estrogen receptor in clinical breast cancer. *J. Steroid Biochem. mol. Biol.*, **43**, 243-247 (1992).
- MIKSICEK, R.J., LEI, Y. and WANG, Y., Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. *Breast Cancer Res. Treat.*, **26**, 163-174 (1993).
- MOALLI, P.A., PILLAY, S., KRETT, N.L. and ROSEN, S.T., Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells. *Cancer Res.*, **53**, 3877-3879 (1993).
- MURPHY, L.C. and DOTZLAW, H., Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. *Mol. Endocrinol.*, **3**, 687-693 (1989).
- MURPHY, L.C., HILSENBECK, S.G., DOTZLAW, H. and FUQUA, S.A., Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin. Cancer Res.*, **1**, 155-159 (1995).
- PFEFFER, U., FECAROTTA, E. and VIDALI, G., Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and MCF-7 cells. *Cancer Res.*, **55**, 2158-2165 (1995).
- QUIGLEY, C.A., EVANS, B.A., SIMENTAL, J.A., MARSCHKE, K.B., SAR, M., LUBAHN, D.B., DAVIES, P., HUGHES, I.A., WILSON, E.M. and FRENCH, F.S., Complete androgen insensitivity due to deletion of exon C of the androgen receptor gene highlights the functional importance of the second zinc finger of the androgen receptor *in vivo*. *Mol. Endocrinol.*, **6**, 1103-1112 (1992).
- RECCHIONE, C., VENTURELLI, E., MANZARI, A., CAVALLERI, A., MARTINETTI, A. and SECRETO, G., Testosterone, dihydrotestosterone and oestradiol levels in postmenopausal breast cancer tissues. *J. Steroid Biochem. mol. Biol.*, **52**, 541-546 (1995).
- SOREIDE, J.A., LEA, O.A., VARHAUG, J.E., SKARSTEIN, A. and KVINNSLAND, S., Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Europ. J. surg. Oncol.*, **18**, 112-118 (1992).
- TEULINGS, F.A.G., VAN GILSE, H.A., HENKELMAN, M.S., PORTINGEN, H. and ALEXIEVA-FIGUSCH, J., Estrogen, androgen, glucocorticoid and progesterone receptors in progestin-induced regression of human breast cancer. *Cancer Res.*, **40**, 2557-2561 (1980).
- TORMEY, D.C., LIPPMAN, M.E., EDWARDS, B.K. and CASSIDY, J.G., Evaluation of tamoxifen doses with and without fluoxymesterone in advanced breast cancer. *Ann. intern. Med.*, **98**, 139-144 (1983).
- TRAPMAN, J., KLAASSEN, P., KUIPER, G.G., VAN DER KORPUT, J.A., FABER, P.W., VAN ROOIJ, H.C., GEURTS VAN KESSEL, A., VOORHORST, M.M., MULDER, E. and BRINKMANN, A.O., Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochem. biophys. Res. Comm.*, **153**, 241-248 (1988).
- WANG, Y. and MIKSICEK, R.J., Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.*, **5**, 1707-1715 (1991).
- WOOSTER, R., MANGION, J., EELES, R., SMITH, S., DOWSETT, M., AVERILL, D., BARRETT-LEE, P., EASTON, D.F. and PONDER, B.A.J., A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome. *Nature (Genet.)*, **2**, 132-134 (1992).

ALTERNATIVE SPLICING OF NEURAL-CELL-ADHESION MOLECULE mRNA IN HUMAN SMALL-CELL LUNG-CANCER CELL LINE H69

C.E.C. MOOLENAAR^{1,2}, Cynthia PIENEMAN¹, Frank S. WALSH², Wolter J. Moor¹ and Rob J.A.M. MICHALIDES^{1,2}

¹Division of Tumor Biology of The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands; and

²Department of Experimental Pathology, Guy's Hospital, London, UK

The neural-cell-adhesion molecule (NCAM) is expressed in all small-cell lung cancers (SCLC) and in approximately 20% of non-small-cell lung tumors (non-SCLC). These NCAM-positive lung tumors have a poor prognosis compared with NCAM-negative tumors. Multiple NCAM protein isoforms are expressed from a single copy gene as a result of alternative splicing and/or post-translational modifications. Therefore, we studied the NCAM isoforms expressed in a human small-cell lung-cancer cell line, H69. NCAM mRNA transcripts of 7.2, 6.7, 4.3 and 4.0 were detected in these cells on Northern blots. Since the various NCAM isoforms may have different biological properties, we performed a more precise examination of NCAM mRNAs using polymerase chain reactions (PCR) with primers flanking the various NCAM exon boundaries. The shortest alternatively spliced sequence that we found was the trinucleotide AAG located between exon 12 and 13 in the so-called hinge region of the NCAM protein. This AAG trinucleotide was present in the majority of the NCAM mRNAs. A second alternatively spliced 30 nt-exon VASE (immunoglobulin-variable domain-like alternatively spliced exon) was present in all NCAM transcript isoforms at the exon 7/exon 8 junction. VASE resulted in the insertion of 10 amino acids into the 4th immunoglobulin-like loop of the NCAM protein. Within the limits of the PCR methodology, no evidence for the presence of mRNA containing exon 15, encoding the glycosyl-phosphatidylinositol-linked (GPI-linked) NCAM isoform in H69 cells was obtained. Considering that H69 cells express 2 major NCAM protein classes (NCAM-180 and NCAM-140), and that the VASE and AAG alternative mRNA splice variants result in minor differences in protein sizes, at least 8 polypeptide isoforms of NCAM might be expressed in H69 cells that contribute to the binding interactions of NCAM.

© 1992 Wiley-Liss, Inc.

NCAM is expressed in lung tumors and its presence correlates with poor prognosis; (Kibbelaar *et al.*, 1991; Mooleenaar *et al.*, 1990). Neural-cell-adhesion molecules (NCAM) are a family of closely related cell-surface sialoglycoproteins involved in homotypic and heterotypic cell-cell interactions. NCAM binds in a homophilic manner, i.e., NCAM proteins bind to one another. The region of the NCAM polypeptide that mediates the NCAM-NCAM interaction, determines also the affinity of NCAM for heparin (Reyes *et al.*, 1990). We have hypothesized that the metastatic potential of SCLC is, at least in part, caused by altered binding properties of NCAM as a result of either alternative splicing or post-translational modifications (Mooleenaar *et al.*, 1990; Kibbelaar *et al.*, 1991).

NCAM undergoes a variety of post-translational modifications, including phosphorylation, sulphation, N-linked glycosylation, and addition of $\alpha(2,8)$ -linked polysialic acid chains (PSA). The latter modification influences NCAM-NCAM binding and might therefore interfere with intercellular adhesion (Rutishauser *et al.*, 1988; Acheson *et al.*, 1991).

Diversity of NCAM proteins is also generated by alternative splicing of NCAM mRNAs (Göddert and Wille, 1988). The human NCAM exists in normal tissues in at least 4 major protein isoforms, i.e., transmembrane polypeptides with MW of 180 and 140 kDa (NCAM-180 and NCAM-140, respectively) and non-transmembrane isoforms of NCAM-115 and NCAM-120 (Bhat and Silberberg, 1988). NCAM-115 and NCAM-120 are respectively secreted or anchored to the cell membrane via covalent linkage to glycosyl-phosphatidylinosi-

tol (GPI) (Gower *et al.*, 1988; Barton *et al.*, 1988). Five different major size classes of mRNAs (7.2, 6.7, 5.2, 4.3, 2.9 kb) encode the NCAM polypeptides in these tissues (Gower *et al.*, 1988). The transcripts are generated from a single-copy gene located on human chromosome 11q23. The NCAM gene consists of at least 20 exons (Fig. 1) (Owens *et al.*, 1987). Exons 0 to 14, coding for the extracellular domain of NCAM, have been found in all NCAM mRNAs thus far identified (numbering system for NCAM exons according to Owens *et al.*, 1987). Exons 15 and 16 encode GPI-linkage to the cell membrane and the transmembrane domain, respectively; the remaining exons encode the cytoplasmic domain of NCAM. In addition to the 15 "common" exons (0 to 14) coding for the extracellular domain, other alternatively spliced exons have been identified, the expression of which is tissue-specific and/or developmentally regulated (Dickson *et al.*, 1987; Small *et al.*, 1988; Thompson *et al.*, 1989; Hemperley *et al.*, 1990). It is assumed that these various NCAM isoforms differ not only in their polypeptide and/or carbohydrate composition, but also in their biological properties (Doherty *et al.*, 1989, 1990; Pöllerberg *et al.*, 1990).

Quantitative and qualitative changes in NCAM expression are observed during embryonic development. Modulation may be necessary for proper tissue segregation, and changes in the regulation of NCAM expression could therefore play a role in tissue morphogenesis and in cell motility (Edelman, 1988). When compared with normal cells, malignant cells most likely have an altered cell surface that causes disruption of normal cellular interactions. NCAM on tumor cells might affect these cellular interactions and thereby influence the invading capacity of tumor cells (Kibbelaar *et al.*, 1991; Roth *et al.*, 1988; Linneman *et al.*, 1989; Andersson *et al.*, 1991). For that reason, a more detailed analysis of NCAM isoforms in small-cell lung carcinoma (SCLC) was performed using the polymerase chain reaction (PCR) technique with primers flanking various NCAM exon boundaries.

MATERIAL AND METHODS

Cell lines and tissues

The small-cell lung-cancer cell lines, H69 and A549, were generous gifts of Dr. D. Carney (Dublin, Ireland) and Dr. A. Jongsma (The Netherlands Cancer Institute, Amsterdam), respectively. Neuroblastoma cell line CHP-212 was kindly provided by Dr. Schlesinger (Schlesinger *et al.*, 1976). The following cell lines were obtained from the ATCC (Rockville, MD): A375, SK-N-SH, U-138 MG, U-373 MG, MCF-7, Ovar-4 and PK-11.

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics.

³Present address: Institut für Mikrobiologie, Heinrich Heine Universität, Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1, Germany.

⁴To whom correspondence and reprint requests should be addressed.

Received: September 4, 1991 and in revised form January 8, 1992.

RNA and Northern blotting

Total RNA was isolated using the LiCl (fetal tissues) or the guanidinium-isothiocyanate technique (normal tissues, cell lines) (Davis *et al.*, 1986). Poly(A)⁺ RNA was isolated by using oligo(dT)-cellulose (Davis *et al.*, 1986). For Northern blotting, poly(A)⁺ RNA was separated on 1% formaldehyde agarose gels, and transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) as described by Maniatis *et al.* (1982). Plasmid probes were radioactively labeled with a random-primed DNA labeling kit according to the instructions of the manufacturer (Boehringer, Mannheim, Germany). Primer probe pVASE (5'-CTCTTGCTTCTCTGTCGAGTCCAC-GA-3') was end-labeled with ³²P-γ-ATP by T4 polynucleotide kinase (Boehringer) in 0.1 M Tris/HCl (pH 9.5), 20 mM MgCl₂, 10 mM DTT, 6% glycerol at 37°C during 30 min. (Davis *et al.*, 1986). Hybridization with plasmid probes or pVASE was performed in hybridization buffer (6 × SSC, 10% dextran sulfate, 0.5% SDS, 0.2% Denhardt solution, 50 μg/ml salmon sperm DNA) at 63°C. After hybridization, filters were washed 3 times in 0.1 SSC, 0.1% SDS at 63°C (for plasmid probes) or in 2SSC at 58°C (pVASE). Blots were exposed to Kodak-X-Omat AR films in the presence of intensifying screens. Before Northern blots were re-probed, they were washed twice in 0.1% SDS at 80°C and exposed to a film for 2 weeks to check for the absence of radioactivity.

cDNA preparations

Randomly primed first-strand cDNA was synthesized from 3 μg of mRNA (cell lines), or 10 μg of total RNA (tissues) by AMV reverse transcriptase (AMV-RT, Boehringer). mRNA was incubated for 60 min at 37°C in 50 mM Tris/HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 2.5 mM DTT, 1 mM each dNTP (Boehringer) in the presence of 200 ng random primers and 25 units of AMV-RT (mRNA⁺). The negative control (mRNA⁻) underwent the same procedure, only AMV-RT was excluded from the reaction mixture. 1/10 (=2 μl) of the reaction mixture was used as a template for each PCR.

PCR

PCRs were performed as described by Innis *et al.* (1990) with minor modifications. Briefly, cDNA templates were amplified in the following conditions in a 70-μl volume: 10 mM Tris/HCl (pH 8.4), 25 mM KCl, 1.5 mM MgCl₂, 140 μM of each dNTP, 700 nM of each primer and 2 U of Taq polymerase (Cetus, Emeryville, CA). Genomic DNA was first denatured at 100°C during 5 min and immediately chilled on ice before it was used as a template in a PCR reaction. dsDNA was dissociated at 90°C for 90 sec, the annealing step was at 55°C for 1 min and the extension step at 70°C for 2.5 min. The reactions were subjected to 30 cycles of amplification.

As a positive control, we used full-length cDNA clones of NCAM-140 (D1, Doherty *et al.*, 1989) or GPI-linked NCAM-120 (λ9.5, Dickson *et al.*, 1987). The following primers were used (nucleotides are given from a 5' to 3' direction):

1a (CTGCAGGTGGATATTGTTCC), 3a (TGATTGTGTGT-GATGTGGTC), 3b (TTGTGTTTCCAGATGATGGT), 5a (TGAATGCCAC-CGCCAACCTC), 5b (GGGAAGCCTTCGGCATCGCA), 7a (ATCACATATG-TAGAAACCA), 7b (CTGATGTTCCGGGTAGAAAGT), 9a (ACATCACCT-GCGAGGTATTT), 9b (GGGGTGTGTAGATCTTGAT), 11a (CCCATCCT-CAAATACAAAGC), 11b (GAATGCCATACTTCTTCACC), 13a (GGGAAC-CCAGTGCACCTAAG), 13b (GTTCACTTTAATAGAGTTTC), 15 (GACAAAGGT-GTAGGATGCAG),

16a (AACGCCAGCCCCACCTCAGG), 16b (GACAT-CACCTGCTACTTCCT), 16c (TTGCCCTCCTCCATGTCCTT), 19a (GAAGTCAA-GACGGTCCCCAA), 19b (ATCATGCTTTGCTCTCGTTC), 19c (GGGTCCCTT-TGTTGCACACT), 19d (GTTTGGCTGTGGGTTTAACC), 19e (AGCTA-GAAAGGGTGACACAC).

Sequence data were obtained from D1 (Doherty *et al.*, 1989) and λ9.5 (Dickson *et al.*, 1987). Part of the PCR products were radioactively end-labeled (see RNA and Northern blotting) and analyzed on a 6% sequencing gel. Due to a difference in electrophoretic mobility of both strands of the PCR product, they sometimes appear as a doublet. For some of the minor bands a second round of PCR amplification was performed on isolated fragments to increase the yield of DNA. They were analyzed by Southern blotting and hybridized to an NCAM-140 cDNA probe (HindIII-BamHI fragment of D1; Doherty *et al.*, 1989) as described by Maniatis *et al.* (1982).

Cloning of PCR products

PCR fragments were purified by gel electrophoresis and phenol/chloroform extraction. Purified bands (7a-9b, 11a-13b and 13a-19e) were ligated into the SmaI-site of pEMBL18 (Promega, Leiden, The Netherlands). Cloned PCR products were sequenced with a DNA sequencing kit according to the manufacturer's instructions (Sequenase 2.0, United States Biochemical Corporation, Cleveland, OH).

RESULTS

PCR

To perform the PCR, we first had to identify the exon boundaries of the human NCAM gene in order to allocate the proper primers. Since the composition of the human NCAM gene is not yet known, we deduced its exon boundaries by comparison of the human NCAM sequence with the chicken NCAM gene. The NCAM gene is highly conserved between the species. A detailed comparison of exon-intron boundaries showed that the organization of the NCAM gene is conserved

TABLE 1 - HOMOLOGY BETWEEN HUMAN AND CHICKEN NCAM cDNAs

Human exon number	Homology to chicken NCAM (%)
0	39 (protein coding sequence: 83%)
1	74
2	75
3	78
4	78
5	68
6	77
7	72
8	74
9	78
10	73
11	75
12	67
13	73
14	72
16	78
17	78
19	51 (protein coding sequence: 76%)

The exon junctions of the human NCAM-140 cDNA (D1) were deduced from the known chicken NCAM exon organization. The exon numbers are according to the numbering system of Owens *et al.* (1987) and are indicated in the first row. The percentage homology at the base pair level is indicated in the second row. Sequence data were obtained from Dickson *et al.* (1987) and Doherty *et al.* (1989) (human NCAM) and Owens *et al.* (1987) (chicken NCAM).

in rat and chicken NCAM (Chen *et al.*, 1990). The homology between chicken and human NCAM protein coding sequences is also very high (from 67% to 83%, see Table I). We now used the known exon organization of the chicken NCAM to deduce the possible position of exon junctions in the human NCAM-140 cDNA clone (D1) (Fig. 1). On the basis of these data, primers were designed and used to determine the splicing pattern of NCAM mRNA in H69 cells (Fig. 1). PCR reactions were performed on randomly primed cDNA with various primer combinations and its products were analyzed on 6% sequencing gels or agarose gels, using D1 and λ 9.5 corresponding with NCAM-140 and NCAM-120 respectively as positive controls.

Primer combination 13a-16c. With this primer combination, we found, in contrast to the 492 bp PCR product of control D1-NCAM (Fig. 2, lane 17), a band with an estimated size of 498 bp in H69 (Fig. 2, lanes 16 and 18). Sequence analysis of the cloned PCR product showed 6 extra nucleotides, [GT-CATG (α)], that were present at position 685 in λ 4.4 (Dickson *et al.*, 1987). α was not located at a predicted exon boundary, but was situated within the trans-membrane coding domain of exon 16. α was found within exon 16 when genomic DNA was analyzed with primer combination 13a-16c (data not shown). We also analyzed several other NCAM-expressing cell lines (A375, Ovar-4, SK-N-SH, A1c-3, CHP-212) for the presence of α , using PCR methods. In all cell lines, only PCR products containing α were detected (data not shown). Sequence comparison between the trans-membrane domains of rat, mouse, chicken and human NCAM again revealed that only control D1-NCAM lacks the 6-bp insert. The absence of introns in exon 16, in spite of the presence of α , excludes the possibility of alternative splicing in this region. The absence of α in D1 is, therefore, due to a cloning artefact or represents a rare genetic polymorphism. Studies by Hemperley *et al.*, 1990, have independently confirmed the presence of α in exon 16 of at least 8 NCAM containing cell lines.

Primer combination 11a-13b. The PCR products of H69 with these primers differed from D1 (Fig. 2, lanes 14 and 15). Sequence analysis of this PCR product revealed an additional trinucleotide, AAG, at the exon 12/exon 13 junction in the H69 mRNA (Fig. 3), resulting in the expression of Gln and Gly instead of Arg. The AAG triplet has been described in human, mouse, rat and chicken NCAM cDNAs, whether or not in combination with other alternatively spliced exons (Andersson *et al.*, 1990; Hemperley *et al.*, 1990; Santoni *et al.*, 1989), and is located in the corresponding hinge region of the NCAM protein (Santoni *et al.*, 1989).

Various other alternatively spliced exons have been described at the exon 12/exon 13 junction (SEC, MSD (Gower *et al.*, 1988; Dickson *et al.*, 1987). Our results show that at the exon 12/exon 13 junction in NCAM transcripts of H69 cells only the trinucleotide AAG is present. Exon SEC (239 bp) is not present, providing evidence that H69 cells do not express a secreted NCAM-SEC isoform.

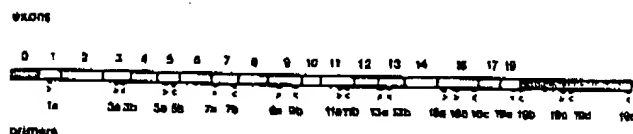


FIGURE 1 - Schematic representation of the exon junctions in the human NCAM-140 cDNA D1 (Owens *et al.*, 1987). The exon junctions of D1 were deduced from the chicken NCAM gene organization by sequence comparison. Above the scheme are the exon numbers, below the scheme, the position and numbers of the various primers. The transcription of exon 15 instead of exons 16 to 19 results in the expression of NCAM-120, the GPI-linked NCAM isoform. The mRNAs containing exon 18 code for the NCAM-180 isoform.

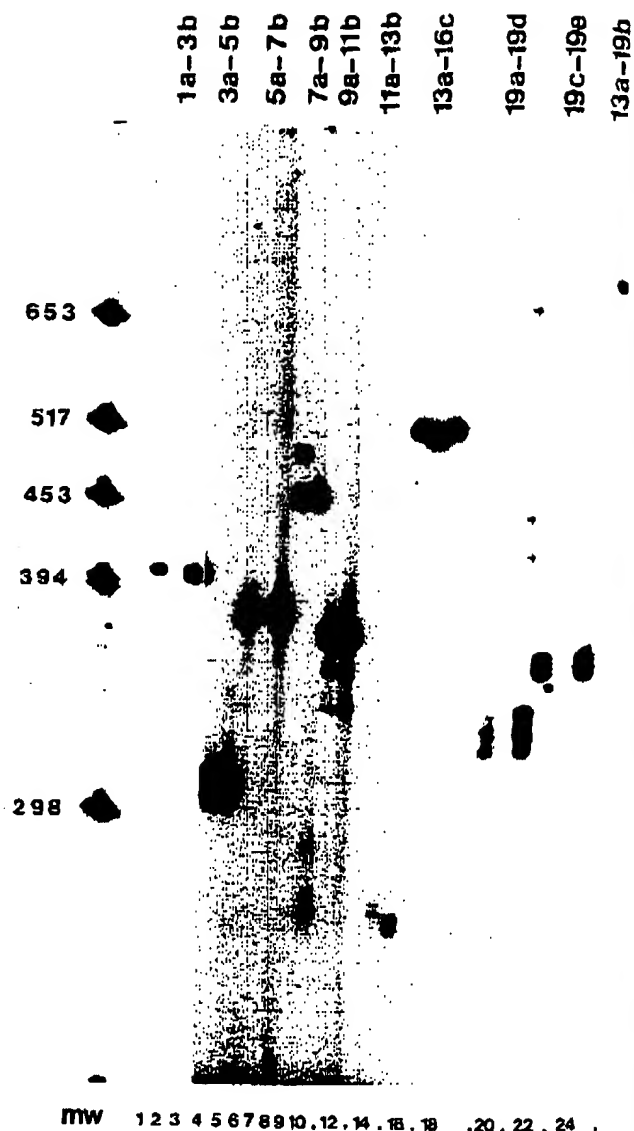


FIGURE 2 - NCAM PCR products in H69. PCR reactions were performed with various primer combinations: primers 1a-3b; lane 1, 2, 3 (+, -, D1), primers 3a-5b; lane 4, 5 (+, D1), primers 5a-7b; lane 6, 7, 8 (+, -, D1), primers 7a-9b; lane 9, 10 (+, D1), primers 9a-11b; lane 11, 12 (+, D1), primers 11a-13b; lane 13, 14, 15 (+, D1), primers 13a-16c; lane 16, 17, 18 (+, D1, +), primers 19a-19d; lane 19, 20, 21 (+, -, D1), primers 19c-19e; lane 22, 23, 24 (+, -, D1), primers 13a, 19b; lane 25 (+). As templates, we used H69 mRNA, whether or not transcribed into cDNA in the presence or absence of AMV-RT (+) or (-) (see "Material and Methods") or D1. On the left, DNA MW marker VI (Boehringer) is given. Primer combinations used are indicated at the top.

GPI-linkage of H69 NCAM. With primer combination 11a-15 we analyzed the presence of an mRNA that can encode a GPI-linked NCAM-isoform in H69 cells. In contrast to the positive control (Fig. 4, lane 5), no amplified product could be detected in H69 when the GPI-specific primer 15 was used (Fig. 4, lanes 3 and 4). The H69 cDNA gave results as expected with primer combination 13a-19b (Fig. 4, lane 1), ensuring that the synthesis of cDNA was correct. We conclude from this experiment that no GPI-linked NCAM isoform is detectable in H69 cells.

Primer combination 7a-9b. In addition to the 448-bp D1 PCR product, a band of 478 bp is found in H69. This alternative splicing event was not observed in all NCAM mRNAs (Fig. 2, lane 9). After sequencing of the cloned 478-bp PCR product,

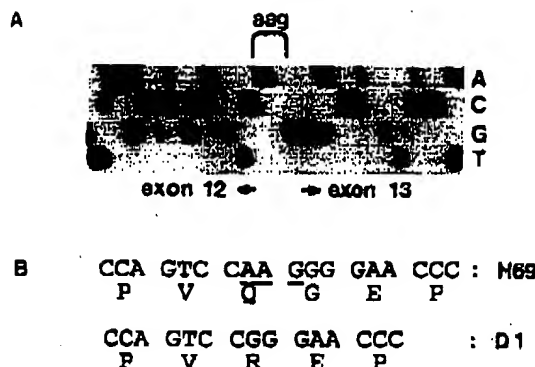


FIGURE 3 - Exon 12/exon 13 junction of NCAM mRNA in H69. (a) Sequencing gel of the 11a-13b PCR product; the position of the trinucleotide AAG is marked (b) The nucleotide and amino acid composition of the exon 12/exon 13 junction of H69 (top) (the trinucleotide AAG is underlined) and D1 (bottom).

we identified a 30-bp VASE exon at the exon 7/exon 8 junction (Fig. 5). The 30-bp VASE exon was first described in NCAM of rat brain (Small *et al.*, 1988), and appears at the amino acid level by comparison with the human VASE sequence fully homologous to the rat VASE sequence.

Primer combinations 19a-19d, 19c-19e. The extra bands indicated in Figure 3, lanes 21, 22 and 24, appeared upon hybridization to NCAM probes to represent PCR artefacts.

VASE expression in other tumor cell lines

VASE was also expressed in human fetal brain (12 weeks of gestation) and in various other tumor cell lines. The tumor cell lines Alc-3 (SCLC), CHP-212, SK-N-SH (neuroblastoma), A375 (melanoma), Ovar-4 (ovarium carcinoma) and U-373 MG (glioblastoma, astrocytoma) all express NCAM on their cell surface (Moolenaar *et al.*, 1990; data not shown). With PCR using primers 7a-9b we could identify the 448-bp band that represents the normal exon 7/exon 8 junction, as well as the 478-bp band that contains the VASE sequence (Fig. 6). The 478-bp band is also visible in Ovar-4 after longer exposure time.

Northern blot analysis of VASE expression

The different NCAM mRNAs are generated by alternative splicing and/or by polyadenylation from a single NCAM gene. Five major NCAM mRNA size classes of 7.2, 6.7, 5.2, 4.3 and 2.9 kb are found in normal human tissues (Gower *et al.*, 1988). To determine with which sub-set of mRNAs VASE is associated, hybridizations were carried out with the pVASE that is complementary to the VASE coding sequence. No NCAM mRNA was detectable in the NCAM-negative cell line PK11 (Fig. 7a, lane 2). At least 4 major mRNA size classes (6.7, 4.3, 4.0 and 2.9 kb) were detectable in the cell lines U-138 MG and A375 (Fig. 7a, lanes 1 and 6). Ovar-4 and CHP-212 expressed the 6.7-, 4.3- and 4.0-kb transcripts (Fig. 7a, lanes 3 and 5). The cell line H69 expressed the 7.2-, 6.7-, 4.3- and 4.0-kb NCAM transcripts (Fig. 7a, lane 4), also other minor transcripts are detectable (indicated by arrows). The upper minor band might be a gel-running artifact, due to the presence of the 28S ribosomal RNA band or is an unidentified new NCAM mRNA size class of 4.5 kb.

When RNA was hybridized to the VASE-specific probe (Fig. 7b), all NCAM transcripts also hybridized to pVASE, indicating that VASE is associated with all of the NCAM mRNAs. Some variation was seen in the VASE expression; the fraction of VASE containing NCAM mRNAs was higher in A375 when compared with U-138, Ovar-4 and CHP-212 (compare Fig. 7a with 7b). Equal amounts of mRNA were

749-

-659

1 2 3 4 5

FIGURE 4 - GPI-linkage of H69 NCAM. PCR reactions were performed with primer combination 13a-19b (lanes 1, 2), or primer combination 11a-15 (lanes 3 to 5). As templates, we used H69 mRNA, whether or not transcribed into cDNA in the presence or absence of AMV-RT (mRNA⁺, lanes 1 and 3, or mRNA⁻, lanes 2 and 4, respectively, see "Material and Methods") or λ9.5, lane 5 (Barton *et al.*, 1988).

loaded in lanes 1, 2, 3, 5 and 6, as was verified by hybridization with a GAPDH-specific probe (data not shown).

DISCUSSION

It is widely accepted that changes on the cell surface are a key event in the process of malignant transformation. These alterations may involve adhesion molecules binding to extracellular matrix components, or interacting with neighbouring cells. Expression of the adhesion molecule NCAM in lung tumors correlates with poor prognosis (Kibbelaar *et al.*, 1991). Since NCAM is expressed in multiple protein isoforms, which may differ in their adhesive properties, we have set out to analyze more precisely the NCAM variants expressed in various types of lung tumor. In this study, we analyzed the NCAM mRNAs in SCLC cell line H69. By Northern blot analysis we observed at least 4 major mRNAs size classes of 7.2, 6.7, 4.3, and 4.0 kb in this cell line. All except the 4.0-kb NCAM transcript are known to be expressed in some normal tissues. Patel *et al.* (1991) have also identified a novel NCAM mRNA transcript in tumor tissues. Further characterization of these possibly tumor-specific NCAM variants is needed.

Expression of the 7.2- and 6.7-kb mRNA, coding for NCAM-180 and NCAM-140 respectively, coincides with the presence of both protein isoforms in H69 cells. The secreted NCAM isoform encoded by a 5.2-kb NCAM mRNA (Gower *et al.*, 1988) is not detected in H69 cells by Northern blotting or by PCR analysis. These results are in line with our previous

exon 7

VASE

exon 8

H69: AAG GCT TCG TGG ACT CGA CCA GAG AAG CAA GAG ACT CTG GAT GGG

K A S W T R P E K Q E T L D G

RAT: AAG GCA TCG TGG ACT CGA CCA GAG AAG CAA GAG ACT CTA GAT GGG

K A S W T R P E K Q E T L D G

D1: AAG ACT CTG GAT GGG CAC ATG GTG GTG CGT TAA GCC

FIGURE 5 - The human VASE sequence. Nucleotide and amino acid composition of the VASE containing exon 7/exon 8 junction in NCAM mRNA in H69 (top) and rat (middle; Small *et al.*, 1988) is compared with D1 (bottom row). The VASE exon is underlined.

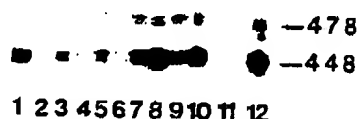


FIGURE 6 - VASE containing mRNAs in tumor cell lines. Amplified products of primer combination 7a-9b were analyzed in various cell lines: D1 (lane 1), H69 mRNA⁻ (lane 2), H69 mRNA⁺ (lane 3), MCF-7 mRNA⁺ (lane 4), A375 mRNA⁺ (lane 5), Ovar-4 mRNA⁺ (lane 6), SK-N-SH mRNA⁺ (lane 7), ALC-3 mRNA⁺ (lane 8), U-373 MG mRNA⁺ (lane 9), CHP-212 mRNA⁺ (lane 10), and in human embryonal brain mRNA⁻ (lane 11), human embryonal brain mRNA⁺ (lane 12) (see also the legend of Fig. 2). PCR product sizes are indicated on the right.

finding that only NCAM-180 and NCAM-140 proteins are present in H69 cells (Moolenaar *et al.*, 1990).

The diversity of NCAM transcripts in SCLC is further illustrated by the presence of a trinucleotide insertion between exon 12 and exon 13. This trinucleotide has been described by others, and is usually observed in combination with other alternatively spliced exons (SEC, MSD; Gower *et al.*, 1988; Dickson *et al.*, 1987; Thompson *et al.*, 1989; Hemperley *et al.*, 1990), but appears from our study and that of Santoni *et al.* (1989) also without these exons. The position of the exon 12/exon 13 junction corresponds with the hinge region of NCAM (Hall and Rutishauser, 1987). The alteration due to this trinucleotide insert, the replacement of Arg by Gln and Gly, is only modest. It therefore remains unclear whether this alteration affects the binding properties of NCAM.

We have identified another alternative splicing event in the region coding for the fourth immunoglobulin-like loop. We found a 30-nt VASE exon inserted at the exon 7/exon 8 junction of all NCAM mRNAs, confirming the findings of Andersson *et al.* (1990) and Small *et al.* (1988) that all NCAM mRNA size classes consist of 2 forms, one with and one without the VASE exon. These authors suggested that expression of the VASE exon correlates with differentiation, since the VASE expression increased during development from 3% in embryonal whole rat brain to 40 to 45% in adult brain (Small *et al.*, 1988). This might also account for the various VASE expression levels in the different cell lines.

One of the early steps involved during the process of metastasis is a change in the adhesive properties of tumor cells (Fidler, 1990). Malignant cells are able to detach from the primary tumor and disseminate to distant sites. Several adhesion molecules that could be involved in this process have been

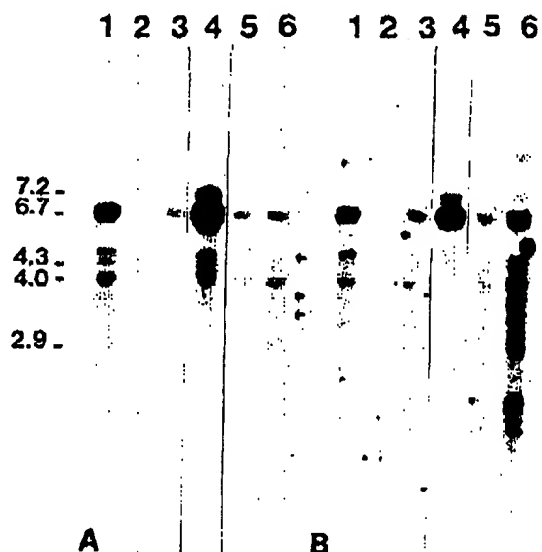


FIGURE 7 - All NCAM RNA transcripts contain VASE. Poly(A)⁺ RNA (10 µg/lane) from U138 (lane 1), PK11 (lane 2), Ovar-4 (lane 3), H69 (lane 4), CHP-212 (lane 5) and A375 (lane 6) were subjected to Northern blot analysis. The filter was probed with a 918-bp PstI fragment of D1 (a), and pVASE (b). mRNA sizes are indicated on the left, the positions of the ribosomal RNAs on the right.

described. The alterations in adhesive properties of tumor cells could be the result of *de novo* expression, reduced expression, or altered expression due to alternative splicing and/or post-translational modifications. NCAM on tumor cells may be involved in this process in various ways: (1) by a partial or total reduction of NCAM, as was found in model systems of the rat and mouse (Linneman *et al.*, 1989; Andersson *et al.*, 1991) and in neuroblastoma cells overexpressing *N-myc* (Axelson and Bernards, 1990); (2) via secretion or release of membrane-bound NCAM in the interstitium. Soluble NCAM could compete for binding to membrane-bound NCAM, and may thereby interfere with NCAM-mediated intercellular adhesion. The absence of the SEC exon and of exon 15 in H69 NCAM mRNAs, however, indicates that H69 cells do not secrete NCAM proteins via this mechanism; (3) via the expression of other alternatively spliced NCAM variants with different binding properties. VASE could be such a relevant NCAM variant. Various functional properties have been suggested for this VASE insertion: duplication of the ho-

homophilic binding site, or interference with functional sites caused by a conformational change (Small *et al.*, 1988). Transfection studies with NCAM cDNAs that contain or lack VASE to test this hypothesis are under way; (4) via post-translational modifications. Attachment of polysialic acid (PSA) on NCAM could interfere with cellular interactions. The highly negative charge of PSA inhibits not only homophilic binding of NCAM, but is also postulated to interfere with interactions of other CAMs with their ligands (Rutishauser *et al.*, 1988; Acheson *et al.*, 1991). We have shown that, as in Wilms' tumors, highly sialylated NCAM is expressed in lung tumors (Moolenaar *et al.*, 1990; Kibbelaar *et al.*, 1989). Further studies on PSA expression in various neuro-endocrine lung-tumor types (SCLC, carcinoids) revealed that the presence of

PSA is associated with the more aggressive types, i.e., atypical carcinoids and small-cell lung carcinomas (data not shown).

In addition to the post-translational modification of tumor NCAM by PSA, we have indicated a second mechanism, alternative splicing, which contributes to the NCAM protein diversity in tumors. The insertion of the AAG trinucleotide and/or the VASE exon may well influence the binding properties of NCAM, as is known for the addition of PSA.

ACKNOWLEDGEMENTS

This study was partially supported by a grant from Centocor Europe BV, Leiden, The Netherlands. We thank Mrs E. Muller and B. van het Hof for technical assistance, and Dr P. Borst for critical reading of the manuscript.

REFERENCES

- ACHESON, A., SUNSHINE, J.L. and RUTISHAUSER, U., NCAM polysialic acid can regulate both cell-cell and cell-substrate interactions. *J. Cell Biol.* 114, 143-153 (1991).
- AKESON, R. and BERNARDS, R., N-myc down-regulates neural cell adhesion expression in rat neuroblastoma. *Mol. Cell. Biol.* 10, 2012-2017 (1990).
- ANDERSSON, A.-M., GAARDSVOLL, H., GILADI, E., DAHL, B. and BOCK, E., Characterization of rat brain NCAM mRNA using DNA oligonucleotide probes. *FEBS J.* 263, 385-388 (1990).
- ANDERSSON, A.-M., MORAN, N., GAARDSVOLL, H., LINNEMAN, D., BJERKVIG, R., LAERUM, O.D. and BOCK, E., Characterization of NCAM expression and function in BT4C and BT4C₊ glioma cells. *Int. J. Cancer* 47, 124-129 (1991).
- BARTON, C.H., DICKSON, G., GOWER, H.J., ROWETT, L.H., PUTT, W., ELSOM, V., MOORE, S.E., GORIDIS, C. and WALSH, F.S., Complete sequence and *in vivo* expression of a tissue-specific phosphatidylinositol-linked N-CAM isoform from skeletal muscle. *Development* 104, 161-173 (1988).
- BHAT, S. and SILBERBERG, D.H., Adult human brain expresses four different molecular forms of neural-cell-adhesion molecules. *Neurochem. Int.* 13, 487-491 (1988).
- CHEN, A., REYES, A. and AKESON, R., Transcription initiation sites and structural organization of the extreme 5' region of the rat neural-cell-adhesion-molecule gene. *Mol. cell. Biol.* 10, 3314-3324 (1990).
- DAVIS, L.G., DIBNER, M.D. and BATTY, J.F., *Basic methods in molecular cloning*. Elsevier, New York (1986).
- DICKSON, G., GOWER, H.J., BARTON, C.H., PRENTICE, H.M., ELSOM, V., MOORE, S.E., COX, R.D., QUINN, C., PUTT, W. and WALSH, F.S., Human muscle neural-cell-adhesion molecule (N-CAM): identification of a muscle-specific sequence in the extracellular domain. *Cell* 50, 1119-1130 (1987).
- DOBATY, P., BARTON, C.H., DICKSON, G., SEATON, P., ROWETT, L.H., MOORE, S.E., GOWER, H.J. and WALSH, F.S., Neuronal process outgrowth of human sensory neurons on monolayers of cells transfected with cDNAs for five human N-CAM isoforms. *J. Cell Biol.* 109, 789-798 (1989).
- DOBATY, P., COHEN, J. and WALSH, F.S., Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. *Neuron* 5, 209-219 (1990).
- EDLMAN, G.M., Morphoregulatory molecules. *Biochemistry*, 27, 3533-3543 (1988).
- FIDLER, I.J., Critical factors in the biology of human cancer metastasis. Twenty-eighth G.H.A. Clowes Memorial Award Lecture. *Cancer Res.* 50, 6130-6138 (1990).
- GORIDIS, C. and WILHELM, W., The three size classes of mouse NCAM arise from a single gene by a combination of alternative splicing and use of different polyadenylation sites. *Neurochem. Int.* 12, 269-272 (1988).
- GOWER, H.J., BARTON, C.H., ELSOM, V.L., THOMPSON, J., MOORE, S.E., DICKSON, G. and WALSH, F.S., Alternative splicing generates a secreted form of N-CAM in muscle and brain. *Cell* 55, 953-964 (1988).
- HALL, A.K. and RUTISHAUSER, U., Visualization of neural-cell-adhesion molecule by electron microscopy. *J. Cell Biol.* 104, 1579-1586 (1987).
- HEMPERLEY, J.J., DEGUIGLIEMO, J.K. and REID, R.A., Characterization of cDNA clones defining variant forms of human neural-cell-adhesion molecule N-CAM. *J. mol. Neurosci.* 2, 71-78 (1990).
- INNIS, M.A., GELFAND, D.H., SNINSKY, J.J. and WHITE, T.J., *PCR protocols. A guide to methods and applications*. Academic Press, San Diego (1990).
- KIBBELAAR, R.E., MOOLENAAR, C.E.C., MICHALIDES, R.J.A.M., BITTER-SUERMANN, D., ADDIS, B. and MOOI, W.J., Expression of the embryonal neural-cell-adhesion molecule N-CAM in lung carcinoma. Diagnostic usefulness of monoclonal antibody 735 for the distinction between small-cell and non-small-cell lung carcinoma. *J. Pathol.* 159, 23-28 (1989).
- KIBBELAAR, R.E., MOOLENAAR, C.E.C., MICHALIDES, R.J.A.M., VAN BODEGOM, P.C., VANDERSCHUEREN, R.G.J.R.A., WAGENAAR, S.S., DINGEMANS, K.P., BITTER-SUERMANN, D., DALESIO, O., VAN ZANDWIK, N. and MOOI, W.J., Neural-cell-adhesion-molecule expression, neuro-endocrine differentiation and prognosis in lung carcinoma. *Europ. J. Cancer* 27, 431-435 (1991).
- LINNEMAN, D., RAZ, A. and BOCK, E., Differential expression of cell-adhesion molecules in variants of K1735 melanoma cells differing in metastatic capacity. *Int. J. Cancer* 43, 709-712 (1989).
- MANIATIS, T., FRITSCH, E.F. and SAMBROOK, J., *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, NY (1982).
- MOOLENAAR, C.E.C., MULLER, E.J., SCHOL, D.J., FIGDOR, C.G., BOCK, E., BITTER-SUERMANN, D. and MICHALIDES, R.J.A.M., Expression of neural-cell-adhesion-molecule-related sialoglycoprotein in small-cell lung-cancer and neuroblastoma cell lines H69 and CHP-212. *Cancer Res.* 50, 1102-1106 (1990).
- OWENS, G.C., EDELMAN, G.M. and CUNNINGHAM, B.A., Organization of the neural-cell-adhesion molecule (N-CAM) gene: alternative exon usage as the basis for different membrane-associated domains. *Proc. nat. Sci. Acad. (Wash.)* 84, 294-298 (1987).
- PATEL, K., KIELY, F., WALSH, F., PHIMISTER, E. and KEMSHEAD, J.T., Potential diagnostic usefulness of tissue-specific NCAMs in differential identification of small round-cell tumours. *Advanc. Neuroblastoma Res.* 3, 275-281 (1991).
- POLLERBERG, G.E., SCHUCHNER, N. and DAVOUST, J., Differentiation-stage-dependent expression of two forms of the neural-cell-adhesion molecule. *Nature (Lond.)* 324, 462-465 (1986).
- REYES, A.A., AKESON, A., BREZINA, L. and COLE, G.J., Structural requirements for neural-cell-adhesion-molecule: heparin interaction. *Cell Regul.* 1, 567-576 (1990).
- ROTH, J., ZUBER, C., WAGNER, P., TAATJES, D.J., WEISGERBER, C., HEITZ, P.U., GORIDIS, C. and BITTER-SUERMANN, D., Re-expression of poly(sialic acid) units of the neural-cell-adhesion molecule in Wilms' tumor. *Proc. nat. Acad. Sci. (Wash.)* 85, 2999-3003 (1988).
- RUTISHAUSER, U., ACHESON, A., HALL, A.K., MANN, D.M. and SUNSHINE, J., The neural-cell-adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science* 240, 53-57 (1988).
- SANTONI, M.J., BARTHELIS, D., VOPPER, G., BONED, A., GORIDIS, C. and WILLE, W., Differential exon usage involving an unusual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. *EMBO J.* 8, 385-392 (1989).
- SCHLESINGER, H.R., GERSON, J.M., MOORHEAD, P.S., MACUIRE, H. and HUMMELER, K., Establishment and characterization of human neuroblastoma cell lines. *Cancer Res.* 36, 3094-3100 (1976).
- SMALL, S.J., HAINES, S.L. and AKESON, R.A., Polypeptide variation in an N-CAM extracellular immunoglobulin-like fold is developmentally regulated through alternative splicing. *Neuron* 1, 1007-1017 (1988).
- THOMPSON, J., DICKSON, G., MOORE, S.E., GOWER, H.J., PUTT, W., KENIMER, J.G., BARTON, C.H. and WALSH, F.S., Alternative splicing of the neural-cell-adhesion-molecule gene generates variant extracellular domain structure in skeletal muscle and brain. *Genes Dev.* 3, 348-357 (1989).

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.